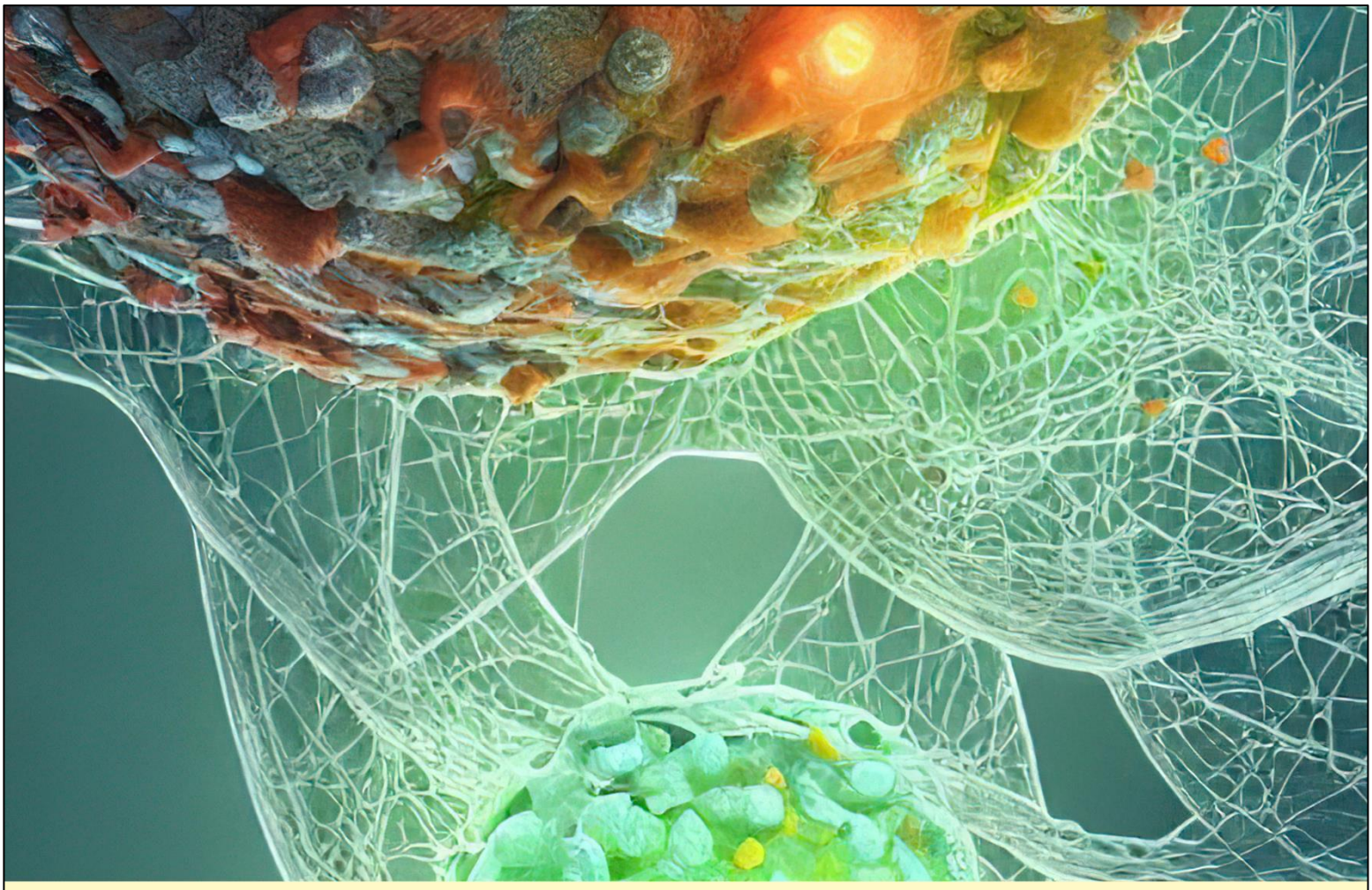

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RESEARCH TRENDS IN MICROBIOLOGY

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PREFACE

Welcome to "Research Trends in Microbiology," an exploration of the dynamic and captivating world of microorganisms and their immense impact on life as we know it. Microbiology, as a scientific discipline, has constantly evolved and revolutionized our understanding of the tiniest forms of life, shaping our perception of the living world and reshaping fields as diverse as medicine, ecology, agriculture, and biotechnology.

This book serves as a comprehensive overview of the latest trends and advancements in microbiology research. We have gathered an array of cutting-edge topics and insights from esteemed researchers and scholars in the field. From the intricate workings of cellular processes to the ecological roles of microorganisms, this volume is designed to illuminate the frontiers of microbiology and inspire curiosity and exploration among readers.

As we venture into the world of microbes, we encounter numerous stories of microbial resilience, adaptability, and even their role as architects of Earth's ecosystem. Their ability to shape the environment and influence macroscopic life is both awe-inspiring and humbling. The collective knowledge presented in this book sheds light on the pivotal role microorganisms play in global biogeochemical cycles, climate regulation, and human health.

Furthermore, we aim to emphasize the importance of research methodologies and technological advancements that have fueled the progression of microbiology. New tools and approaches, such as high-throughput sequencing, single-cell analyses, and gene-editing technologies, have expanded our capacity to investigate microbial communities and understand their complex behaviors and interactions.

This book is not merely a snapshot of present knowledge but also a glimpse of the fascinating directions in which microbiology is headed. The rapid pace of discovery and innovation in this field ensures that new breakthroughs are continuously challenging our existing perspectives.

We extend our gratitude to the contributing authors, whose dedication to scientific inquiry has enriched this compilation. Their contributions will undoubtedly inspire current and future generations of researchers to unravel the mysteries of the microbial world and harness its potential for the betterment of humanity and the environment.

Editors

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BACTERIOCIN PRODUCTION FROM LACTIC ACID BACTERIA - A REVIEW

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Abstract:

Bacteriocins are biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species. Numerous strains of bacteriocin producing *Lactobacillus plantarun* have been isolated in the last two decades from different ecological niches including meat, fish, fruits, vegetables, milk and cereal products. Several of these have been characterized and the amino acid sequence determined. Different aspects including isolation and identification of bacterial strains, screening of isolates for antibacterial activity, effect of pH, temperature, NaCl concentration and organic solvents on bacteriocin production, purification and characterization of bacteriocin were studied.

Keywords: Lactic Acid Bacteria, Bacteriocins, *Lactobacillus plantarum*

Introduction:

A great number of Gram positive bacteria and Gram negative bacteria produce during their growth, substances of protein structure (either proteins or polypeptides) possessing antimicrobial activities, called bacteriocins (Zacharof and Lovitt, 2012). *Lactobacillus* is a diverse group of microorganisms consisting of a number of different species. They are nonspore formers, Gram positive rods ranging from 0.5-1.2x 1-10 µm in size and produce lactic acid as a fermented end product (Aasen *et al.*, 2000; Karthikeyan and Santosh, 2009). The genus comprises over 25 species. Some are homofermentative and some are heterofermentative. Some antibacterial proteins (bacteriocins) from lactic acid bacteria are popular (Nistin). Bacteriocins are bacterial origin proteinaceous compounds lethal to other bacteria. Generally, bacteriocins are named according to the genus or species of the bacterial strain that produces them. For example, plantaricin is produced by *Lactobacillus plantarun* (Joerger *et al.*, 2000; Karthikeyan and Santosh, 2009). To avoid the use of chemical preservatives in food, bacteriocins produced by lactic acid bacteria have received much attention during recent years for their possible application as bio preservatives in food (Karthikeyan and Santosh, 2009). The antimicrobial peptides produced by *Lactobacillus plantarum*,inhibits the growth of number of food spoilage bacteria (Reenen *et al.*, 1998).

In food industry, bacteriocins can be applied in the food industry as natural preservatives. The use of LAB and of their metabolic products is generally considered as safe (GRAS, Grade One). The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient (And and Hoover, 2003).

Most important bacteriocins produced by *Lactobacilli*:

Sr. No.	Bacteriocin	Bacteriocin Producing Strain
1	Lactacin F	*L. johnsonii* spp.
2	Lactocin 705	*L. casei* spp.
3	Lactoccin G	*L. lactis* spp.
4	Lactococcin MN	*Lactococcus lactis* var *cremoris*
5	Nisin	*Lactococcus lactis* spp.
6	Leucocin H	*Leuconostoc* spp.
7	Plantaricin EF, Plantaricin S	*L. plantarum* spp

Bacteriocins can be applied on a purified or on a crude form or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (starter culture). The incorporation of a bacteriocin producing strain has the disadvantage of the lack of compatibility between the bacteriocin producing strain and the other cultures required for fermentation. Furthermore, bacteriocins could be combined with other antimicrobial compounds such as sodium acetate and sodium lactate resulting in enhanced inactivation of bacteria. Bacteriocins can also be used to improve food quality and sensory properties, for example increasing the rate of proteolysis or in the prevention of gas blowing defect in cheese. Another application of bacteriocins is bioactive packaging, a process that can protect the food from external contaminants. For instance, the spoilage of refrigerated food commonly begins with microbial growth on the surface that reinforces the attractive use of bacteriocins being used in conjunction with packaging to improve food safety and self-life. Bioactive packaging can be prepared by directly immobilizing the bacteriocin to the food packaging or by addition of a sachet containing the bacteriocin into the packaged food, which will be released during storage of the food product. The gradual release of bacteriocins from a packaging film on the food surface may have an advantage over dipping and spraying foods with bacteriocins, because antimicrobial activity may be lost or reduced due to inactivation of the bacteriocins by food components or dilution below active concentration due to migration into the foods (Ross *et al.*, 2002).

However, it has been proven that a bacteriocin alone in a food is not likely to ensure complete safety; especially in the case of Gram negative bacteria this has been apparent. Then the use of bacteriocins has to be combined with other technologies that are able to disrupt the cellular membrane so bacteriocins can kill the pathogenic bacteria **(**Daw and Falkiner, 1996). For example, the use of non-thermal treatments such as pulsed electric field (PEF) is advantageous as it does not have any effect on food functionality and nutritional qualities. This technique may not be financially viable when used alone, but in lower levels and combined with other treatments such as bacteriocins may be highly effective.

There are several methods to prepare packaging films with bacteriocins. One method is to incorporate bacteriocin directly into polymers for example incorporation of nisin into biodegradable protein films. The incorporation of nisin or any other bacteriocin can be achieved through heat press and casting into films made from soy proteins or corn zein. Another method is to coat or adsorb bacteriocins to polymer surfaces; examples include nisin methylcellulose coatings for polyethylene films for the use on poultry meat, adsorption of nisin on polyethylene,

ethylene, vinyl acetate, polypropylene, polyamide, polyester acrylics and polyvinyl chloride (Deegan *et al.,* 2006).

Isolation and identification of bacterial strains:

Lactobacillus plantarum was isolated from the guts of dead strimp using the method of Todorov and Dicks (2004). One gram of strimp gut was homogenized in 99 ml of normal sterile physiological saline to form a uniform homogenate and then used for the isolation purpose.1 ml of the gut homogenate was mixed with 9 ml of normal sterile physiological saline and serially diluted up to 10^{-6}. One ml of the last dilution was inoculated into the sterilized plates of de Man, Ragosa and Sharp (MRS) agar supplemented with 50 µg/liter of Natomycin.The culture was then incubated at 37^0C for 72-96 h. After incubation, isolates were identified by observing their morphological, physiological and biochemical characteristics as described by Michael (1981), Karthikeyan and Santosh (2009). The biochemical characteristics observed were gas production, sugar fermentation, resistance to biliary salts and pH determination. The bacteriocin producers were isolated from traditional fermented foods as curd, pickle, idli-batter and butter. The samples for the lactic acid bacteria were inoculated in Man Rogosa Sharpe (MRS) broth at 37^0C for 24 hours. The enriched cultures were grown on MRS agar (Sekar *et al.,* 2013).

The bacteriocin producers were isolated from radish outer surface and putting them on MRS broth. The bacterial growth was diluted, plated on MRS agar and grown at 30^0C for 24 hours (Yildirim and Johnson, 1998).

Screening of isolates for antibacterial activity:

Lactobacillus plantarum isolates were screened for antibacterial activity using the agar spot method. An aliquot of 10 µl cell free culture fluid of *Lactobacillus plantarum* was spotted onto agar plates seeded with indicator organism (*Lactobacillus bulgaricus, Salmonella typhimurium, Bacillus subtilis, Staphylococcus aureus, Salmonella paratyphi B, Escherichia coli, Klebsiella* species, *Serratia marsescance, Pseudomonas aerogenosa* and *Vibrio cholera*). After incubation (37^0C for 24 hours), the plates were examined for the development of 2mm clear zone around the spot (Karthikeyan and Santosh, 2009**;** Reenen *et al.,* 1998). The pathogenic indicators were subcultured in Luria Bertani broth to get maximum growth (Sambrook *et al.,* 1989). These were serially diluted in sterile water up to 10^{-5}**.** These diluted cultures were poured on sterile MRS agar plates and incubated at 37^0C for 16 hours till colony formation. Then plates were laid with MRS soft agar inoculated with lactic acid bacteria and incubated at 37^0C for 24 hours and observed for zone of inhibition (Sekar *et al.,* 2013). To determine the antibacterial activity, sandwich overlay method was used (Mayr-Harting *et al.,* 1972). The indicators tested were *Escherichia coli* ATCC 11775, *Lactobacillus plantarum* NCDO 955, *Listeria monocytogenes* ATCC 19113 and *Bacillus cereus* ATCC 232. Colonies showing inhibition were collected and isolated. Identification of isolated strains was performed using BIOLod, Strep-API-20, API 50CHL and fatty acid profile (MIDI) identification systems.MRS broth was used for the production of antimicrobial agents (Yildirim and Johnson, 1998).

Effect of pH, temperature, NaCl concentration and organic solvents on bacteriocin production:

To determine the effect of pH on bacteriocin production, six batches of 100 ml MRS broth were prepared and adjusted the pH to 1.2.3.4.5 and 6 respectively with 6 M HCl or 6M

NaOH and then autoclaved. The flasks were inoculated with 2% v/v of 18 hours old culture of *Lactobacillus plantarum* and incubated at 30^{O}C for 20 hours without aeration. After incubation, bacteriocin titre was assessed (Karthikeyan and Santosh, 2009). The culture supernatants were adjusted to various ranges from 4 to 8 at room temperature for a definite period and the activity was determined against indicator organisms (Sekar *et al.*, 2013).

To determine the effect of pH on activity of bacteriocin, dried crude bacteriocin preparations at a concentration of 50 mg/ml were dissolved in deionized water. These samples were adjusted with sterile 0.1N NaOH or 0.1N HCl to different pH values between 2 to 10 and assayed for activity with appropriate controls (Yildirim and Johnson, 1998). These samples were maintained for: (1) 2 h at 25^{0}C, (2)24h at 25^{0}C, (3)20 min at 90^{0}C. Then they were adjusted to pH 7.0 and assayed for activity with appropriate controls. For the determination of effect of temperature on bacteriocin production, 100 ml each of sterilized MRS broth in six different sets of flasks were inoculated with 2% v/v of 18 hours old culture of *Lactobacillus plantarum* and incubated without aeration at various temperatures as 10, 20, 30, 40, 50 and 60^{0}C for 20 hours. After incubation, bacteriocin titre was assessed (Bhunia *et al.*, 1988). Yildirim and Johnson (1998) also studied the effect of temperature on bacteriocin production.

The samples (2 ml;10 mg/ml) of crude bacteriocin were heated at 90^{0}C for 15, 30 or 60 min or autoclaved at 121 OC for 15 min, cooled and assayed for the activity. The stability of bacteriocin was determined. For this, the supernatant of LAB was heated at various temperatures at constant time and bacteriocin activity was directed against indicator organisms (Sekar *et al.*, 2013). The effect of NaCl concentration on bacteriocin production was determined by means of preparing ten sets of 100 ml MRS broth, their salt concentration was from 0.1-1.0% respectively. The flasks were inoculated with 2% v/v of 18 hours old culture of *Lactobacillus plantarum* and incubated without aeration at 37^{0}C for 20 hours without aeration. After incubation, bacteriocin titre in the medium was determined as described by Todorov and Dicks (2004).

Yildirim and Johnson in 1998 determined the effect of organic solvents, freeze dried crude bacteriocin was dissolved at a final concentration of 10 mg/ml in 10% w/v of formaldehyde, chloroform, acetone, 2-propanol or 25% w/v of ethyl alcohol, hexane, isobutanol methanol or ethyl ether. Samples were incubated at 25OC for 1 h and solvents evaporated in a centrifugal concentrator. Dried samples were reconstituted with sterile deionized water to their original volumes and assayed for the antimicrobial activity (Bhunia *et al.*, 1988).

Preparation of crude bacteriocin:

Lactobacillus lactis subsp. *cremoris* R was inoculated in MRS broth at a rate of 1%(V/V). After incubation period of 22 h at 30^{0}C. The supernatant was collected by centrifugation at 22100g for 30 min. The obtained supernatant was passed through sterile filters (0.45 and 0.22 µm pore size).and precipitated with 70% ammonium sulphate. The precipitate was dissolved in 50 ml deionized water and dialyzed through a 1000 molecular weight cut off dialysis membrane (Spectrum Houston, TX, USA) against deionized water for 48 hours (Yildirim and Johnson, 1998).

Purification and characterization of bacteriocin:

Partial purification of bacteriocin by adsorption on to producer cells: *Lactobacillus lactis* subsp. *cremoris* R grown in MRS broth at 30^{0}C for 22 h was adjusted to a pH range of 3-9 with a

combination of 5 mmol l^{-1} phosphoric acid or 5 mmol l^{-1} NaOH. Then the samples were heated to 85^0C for 25 min to kill cells and mixed using a magnetic stirrer overnight at 4^0C. Cells were removed by the centrifugation at 22100g for 30 min. In the supernatant fluid, bacteriocin activity was assayed to calculate the amount of bacteriocin not adsorbed onto cells. To get the adsorbed bacteriocin, cells were washed with 5 mmol l^{-1} sodium phosphate of pH 6.5, resuspended in 50 ml of 100 mmol l^{-1} NaCl at pH 2.0 (adjusted with 5% phosphoric acid) and stirred overnight at 4^0C. Centrifugation was performed at 22100g for 30min and supernatant fluid containing bacteriocin were filter sterilized (0.22 µm pore size), dialyzed through a 1000 molecular weight cut-off dialysis bag (Spectrum against distilled water at 4^0C) for 24 hours and then freeze-dried (Bhunia *et al.,* 1988; Yang *et al.,* 1992).

Two methods were also used by Micher *et.al.* in 1980 as suggested below:

1) Ammonium salt precipitation and ion exchange chromatography (Yang *et al.,* 1992). For Ammonium salt precipitation, various concentration of Ammonium Sulphate (10, 20, 30, 40, 50 and 60%) were added to 10 ml of crude bacteriocin in different test tube sets. Then allowed for the precipitation for 24 hours. Then the mixture was centrifuged at 5000 rpm for 10 minutes and the precipitate was resuspended in 25 ml of 0.05M phosphate buffer. The mixture was stirred for 24 hours at 4^0C.Then the suspension was dialyzed in a tubular cellulose membrane (1000 cut off) against 2liter distilled water for 24 hours. After dialysis, the bacteriocin titre was determined.

2) Ion-exchange chromatography- The obtained dialysate was used for the purification by cation exchange column (DEAE cellulose column) and eluted by a linear gradient from citrate phosphate buffer ranging from citrate phosphate buffer ranging from pH 2.6 to 7.0 Bradford method will be used to determine the protein content.

All the bacteriocin producers were grown in MRS broth for 12 hours. Then it was stabilized at room temperature for one hour. After one hour, it was subjected to cell mass recovery by centrifugation for 10 minutes at 12000 rpm. The bacteriocin present in the supernatant further subjected to ultrafiltration and this was accomplished by minimizing the pore size by treating the ordinary filter paper which is with the pore size more than 1 µm. To concentrate, the filtrates were precipitated with Ammonium Sulphate (40% saturation) and held over night at 7^0C with gentle stirring. The samples were centrifuged at 8000 rpm for 15 minutes. The bottom pellets were resuspended in 2 ml 50 mM phosphate buffer. The concentrated bacteriocins were again recoverd by solvent extraction method. To the concentrated bacteriocins, 15 volumes of Chloroform/Methanol mixture (2/1, V/V) was added and kept at 4^OC for one hour. The formed precipitate was centrifuged at 10000 rpm for 30 minutes and pellets were collected and resuspended in 3 ml ultra pure water (Sekar *et al.,* 2013).

Molecular weight of bacteriocin determination:

Molecular weight of partially purified bacteriocin was determined using a 16% tricine-SDS-PAGE gel (Schagger and Von Jagov, 1987). At the end of electrophoresis, the gel with duplicate samples were sliced into two pieces. One gel was stained with Coomassie Blue G-250 for 1h and destained. The other half was stained with Coomassie Blue for 30 min and destained for 1.5h or until the background stain disappeared. After the gel was equilibrated in changing deionized water for 3h, it was placed carefully on a pre-poured MRS agar surface. The gel was

seeded with 30 ml tempered MRS soft agar (0.8% agar) containing *L. plantarum* NCDO 955 as an indicator organism and incubated t 30^0C for 24 hours (Yildirim and Johnson, 1998).

The molecular weight was also determined by 15% Sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Comassie Brilliant Blue R-250. Range molecular markers (29-200 kDa) with five polypeptides were used as markers (Ragaram *et al.*, 2010).

Molecular size of the bacteriocin determination:

Molecular size of the bacteriocins can be determined by tricine-SDS-PAGE according to the described by Schagger and Von Jagow (1987).

Conclusion:

Bacteriocins are produced from bacteria. These bacteriocins are proteinaceous in nature and lethal to other bacteria. The use of LAB and of their metabolic products is generally considered as safe. These are used in the food industry as natural preservatives. The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient. Today to avoid the use of chemical preservatives in food, bacteriocins produced from lactic acid bacteria have received much attention.

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References:

1. Aasen, I. M., Moretro, T., Katla, T., Axelsson, L., and Storro, I. (2000). Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Applied Microbiology and Biotechnology*, *53*, 159-166.
2. And, H. C., and Hoover, D. G. (2003). Bacteriocins and their food applications. *Comprehensive reviews in food science and food safety*, *2*(3), 82-100.
3. Bhunia, A. K., Johnson, M. C., and Ray, B. (1988). Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. *Journal of Applied Bacteriology*, *65*(4), 261-268.
4. Daw, M. A., and Falkiner, F. R. (1996). Bacteriocins: nature, function and structure. *Micron*, *27*(6), 467-479.
5. Deegan, L. H., Cotter, P. D., Hill, C., and Ross, P. (2006). Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International dairy journal*, *16*(9), 1058-1071.
6. Joerger, R. D., Hoover, D. G., Barefoot, S. F., Harmon, K. M., Grinstead, D. A., and Nettles-Cutter, C. G. (2000). Bacteriocins. *Encyclopedia of microbiology*, *1*, 383-397.
7. Karthikeyan, V., and Santosh, S. W. (2009). Isolation and partial characterization of bacteriocin produced from *Lactobacillus plantarum*. *African Journal of Microbiology Research*, *3*(5), 233-239.
8. Macher, B. A., and Klock, J. C. (1980). Isolation and chemical characterization of neutral glycosphingolipids of human neutrophils. *Journal of Biological Chemistry*, *255*(5), 2092-2096.

9. Michael, T. (1981). Family Lactobacillaecea. *The prokaryotes. Blackwell Press, New York, NY, USA*, 609-619.
10. Mohankumar, A., and Murugalatha, N. (2011). Characterization and antibacterial activity of bacteriocin producing *Lactobacillus* isolated from raw cattle milk sample. *International Journal of Biology*, *3*(3), 128.
11. Rajaram, G., Manivasagan, P., Thilagavathi, B., and Saravanakumar, A. (2010). Purification and characterization of a bacteriocin produced by *Lactobacillus lactis* isolated from marine environment. *Advance Journal of food science and technology*, *2*(2), 138-144.
12. Reenen, V., Dicks, and Chikindas. (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by Lactobacillus plantarum. *Journal of applied microbiology*, *84*(6), 1131-1137
13. Ross, R. P., Morgan, S., and Hill, C. (2002). Preservation and fermentation: past, present and future. *International journal of food microbiology*, *79*(1-2), 3-16.
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (No. Ed. 2). Cold spring harbor laboratory press.
15. Sankar, N. R., Priyanka, V. D., Reddy, P. S., Rajanikanth, P., Kumar, V. K., and Indira, M. (2012). Purification and characterization of bacteriocin produced by *Lactobacillus plantarum* isolated from cow milk. *Int J Microbiol Res*, *3*(2), 133-137.
16. Schägger, H., and Von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical biochemistry*, *166*(2), 368-379.
17. Sekar K.V., S. Bharathi, andYogesh B. (2013). Characterization of bacteriocins from the isolates of traditional fermented foods. *Asia Pacific Journal of Research*,1 (1X),27-36.
18. Todorov, S. D., Vaz-Velho, M., and Gibbs, P. (2004). Comparison of two methods for purification of plantaricin ST31, a bacteriocin produced by *Lactobacillus plantarum* ST31. *Brazilian Journal of Microbiology*, *35*, 157-160.
19. Yang, R., Johnson, M. C., and Ray, B. I. B. E. K. (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and environmental microbiology*, *58*(10), 3355-3359.
20. Yildirim, Y., and Johnson, J. (1998). Detection and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* R isolated from radish. *Letters in applied microbiology*, *26*(4), 297-304.
21. Zacharof, M. P., and Lovitt, R. W. (2012). Bacteriocins produced by lactic acid bacteria a review article. *Apcbee Procedia*, *2*, 50-56.

EFFECTS OF DIFFERENT SPECIES OF *TRICHODERMA* AND ITS GENES ON PLANTS- HOST MICROBE INTERACTION

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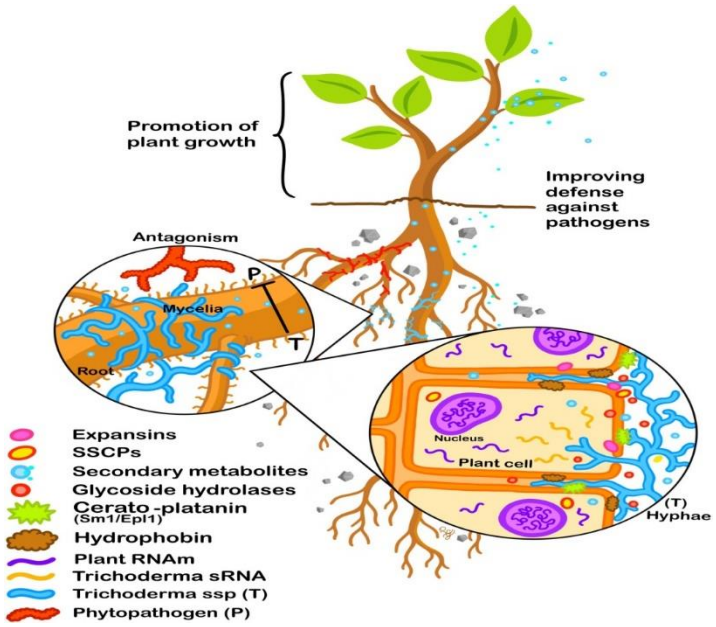
Abstract:

A genus of fungi called *Trichoderma* (teleomorph Hypocrea) is present in various habitats. *Trichoderma* spp.'s highly effective antagonistic and mycoparasitic activity can prevent plant pathogens in the soil, reducing the severity of plant diseases. Furthermore, as recent study has shown, some *Trichoderma* strains can interact directly with roots, enhancing plant growth capacity, disease resistance, and stress tolerance. This book chapter outlines the key facts about the interaction between *Trichoderma* and plants, their molecular communication, and the significant alterations the helpful fungus caused in the plant. Additionally, efforts are made to improve plant tolerance and resistance to a variety of stressors by expressing *Trichoderma* genes in the plant genome.

Introduction:

The quest to improve agricultural quality and output has resulted in an overuse of artificial fertilizers that has seriously polluted the environment. One possibility for maintaining high production with no negative impact on the environment is the use of biofertilizers and biopesticides. Numerous fungus and bacteria from the soil can invade the roots of plants and may benefit the plant. Other plant-growth-promoting rhizobacteria (PGPR) and fungi, such as *Trichoderma* spp. and *Piriformospora indica*, can boost plant development by squelching plant diseases in addition to the traditional mycorrhizal fungi and Rhizobium bacteria [1]. The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) act as signaling mediators in a signaling network that allows the two partners to recognize each other through chemical means. The signal transduction pathway through SA accumulation is seen in the systemic acquired resistance (SAR) generated by attack by pathogens, and JA and ET have been reported as signal transduction molecules for induced systemic resistance (ISR) due to the influence of beneficial bacteria. A genus of fungi called *Trichoderma* (teleomorph Hypocrea) is present in various habitats. Some strains have a high antagonistic and mycoparasitic capability, which they use to prevent plant pathogens, primarily in the soil or on plant roots, hence reducing the severity of plant diseases. We now have a clearer knowledge of how mycoparasitism developed in a common *Trichoderma* ancestor as a way of life for the genus according to the recent comparative genome sequence analysis of two recognized biocontrol species, *Trichoderma atroviride* and *Trichoderma virens* [2]. The availability of nutrients derived from roots and the existence of fungal prey may have been the main draws for the ancestors of *Trichoderma* to settle in the

rhizosphere and promote the evolution of beneficial interactions with plants [3]. Numerous plant pathogens, including fungi, oomycetes, bacteria, and viral diseases, have been reported to be controlled by *Trichoderma* by inducing ISR or regional resistance. According to research by [4], some *Trichoderma* rhizosphere-competent strains can directly affect plants, enhancing their growth potential, nutrient uptake, fertilizer use efficiency, percentage and rate of seed germination, and stimulation of plant defenses against biotic and abiotic damage. In recent years, an increasing number of studies have contributed to unravelling the molecular basis of the plant–*Trichoderma* dialogue and the beneficial effects of *Trichoderma* to plants. This book chapter summarizes the key discoveries regarding the direct *Trichoderma*-plant interaction and the initiatives made to increase plant tolerance and resistance to a wide variety of challenges by expressing *Trichoderma* genes in the plant genome fig-1[5].



Colonization of *Trichoderma spp.*

Numerous root ecosystems contain different *Trichoderma* strains. Similar to mycorrhizae, the highly hydrated polysaccharides of the mucigel layer secreted by the roots and the mono- and disaccharides released by plant roots into the rhizosphere promote fungal growth. According to research, *Trichoderma* cells use plant-derived sucrose as a significant resource to help with root colonization, the coordination of defense systems, and an improved rate of leaf photosynthesis. Strains must be able to colonize plant roots in order to stimulate plant growth and offer infection prevention. Colonization necessitates the capacity for recognition and stick to plant roots, get within the plant, and endure poisonous metabolites the host plant makes in response to invasion. In *Trichoderma*, hydrophobins, which are tiny hydrophobic proteins of the outermost cell wall layer that cover the fungal cell surface, and expansin-like proteins associated with cell wall formation can facilitate adhesion to the root surface. The class I hydrophobin TasHyd1, which is produced by *Trichoderma asperellum*, has been demonstrated to aid in the colonization of plant roots, potentially by strengthening its adhesion to the root surface and

shielding the hyphal tips from plant defense chemicals [6] and the swollenin TasSwo, an expansin-like protein with a cellulose-binding domain able to recognize cellulose and modify the plant cell wall architecture, facilitating root colonization.

Defence role played by *Trichoderma*

Plants have an immune system that is able to recognize motifs or domains with conserved structural properties typical of entire species in addition to naturally occurring physical and chemical barriers groups of bacteria, namely the pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), but not in their host. Plant responses that are generated by MAMP happen quickly and briefly. Ion fluxes across the plasma membrane, the production of ROS, nitric oxide, ET, as well as subsequent callose deposition and antimicrobial chemical formation, are all examples of early MAMP responses. Flagellin and lipopolysaccharides are just two examples of the many MAMPs that have been found for PGPR. However, it has also been demonstrated that secreted substances including antibiotics, biosurfactants, and volatile organic compounds can also cause systemic resistance. MAMPS are produced by productive *Trichoderma* strains.

Effector produced by *Trichoderma*	**Species**	**Role**	**Refrences**
Xylanase Xyn2/Eix	*T. viride*	A xylanase that elicits ET biosynthesis and hypersensitive response in tobacco leaf tissues	[7]
Swollenin TasSwo	*T. asperelloides*	Expansin-like protein with a cellulose-binding domain capable of stimulating local defence responses in cucumber roots and leaves and affording local protection against B. cinerea and P. syringae	[8]
Alamethicin (20mer peptaibol)	*T. viride*	Elicitation of JA and SA biosynthesis in lima bean	[9]
Trichokonin (20mer peptaibol)	*T. pseudokoningii*	Induces the production of ROS, the accumulation of phenolic compounds at the application site and virus resistance in tobacco plants through multiple defence signalling pathways	[10]
6-Pentyl-a-pyrone, harzianolide and harzianopyridone	Various	Low-concentration metabolite activating plant defence mechanisms and regulating plant growth in pea, tomato and canola	[11]

Plant leaves' proteome and transcriptome are adversely impacted as a result of *Trichoderma* root colonization and MAMP interaction. According to several authors,

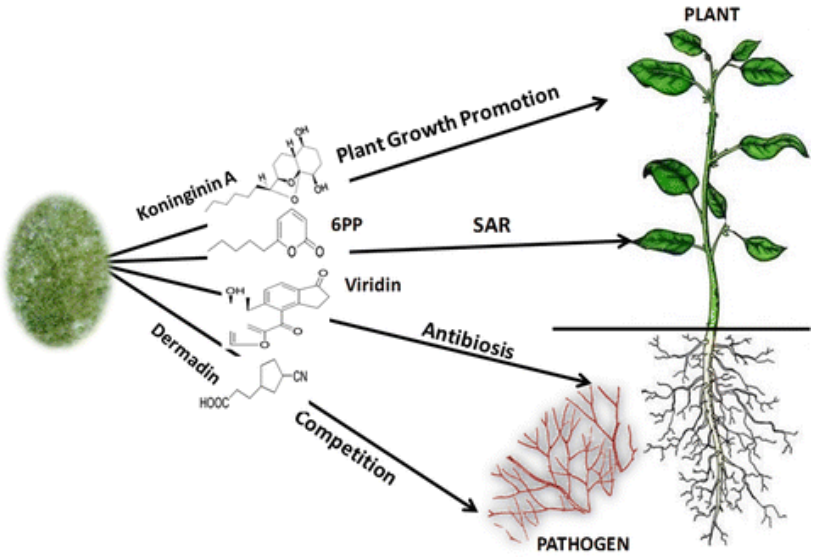
Trichoderma-mediated ISR against *Colletotrichum graminicola* in maize is caused by the JA/ET signaling pathway, just like PGPRISR; *Trichoderma* treatment of JA/ET-deficient Arabidopsis genotypes increases susceptibility to *Botrytis cinerea*; and (iii) ISR triggered by PGPR and increases susceptibility to Botrytis. The production of plant responses in the *T. asperellum*-cucumber interaction, however, is a time- and concentration-dependent process, according to other investigations. Occurrence, and during the first few hours after contact, a rise in SA and peroxidase activity is seen. In fact, following the inoculation of large densities of *Trichoderma*, a systemic rise in SA and JA levels was seen. Several scientists also noted that JA/ET and SA were necessary for the potato's defense response against *Rhizoctonia solani* when it was challenged with *T. harzianum.* Recent findings include: (i) the colonization of Arabidopsis roots by *T. atroviride* induces a delayed and overlapping expression of the defence-related genes of the SA and JA/ ET pathways against biotrophic and necrotrophic phytopathogens, both locally and systemically ; (ii) *Trichoderma* is able to trigger a long-lasting upregulation of SA gene markers in plants unchallenged by pathogens, although when plants are infected by a pathogen such as *B. cinerea*, the pretreatment with *Trichoderma* may modulate the SA-dependent gene expression and, soon after infection, the expression of defence genes induced through the JA signal transduction pathway occurs, causing ISR to increase over time; and (iii) colonization of Arabidopsis root by *T. asperellum* produces a clear ISR through an SA signalling cascade, and both the SA and JA/ET signalling pathways combine in the ISR triggered by cell-free culture filtrates of *Trichoderma*.

***Trichoderma* (microbe) host interaction**

A network of hormone-signaling pathways links plant immunity and development (Pieterse *et al.,* 2009). The key defense players SA, JA, and ET interact with other hormones such as abscisic acid, which is frequently linked to plant development and abiotic stress, IAA, which is linked to plant growth and lateral root development, and gibberellins, which regulate the breakdown of growth-repressing DELLA proteins to control plant growth. In *Trichoderma*, the ACCD activity lowers the amount of ACC that is readily available for ET production. By boosting the breakdown of DELLA proteins, reductions in ET stimulate plant growth through gibberellin signaling. Moreover, gibberellins may control the onset of JA- and SA-dependent defence responses of the plant through the regulation of DELLA protein degradation. ET and IAA in the roots can reciprocally regulate each other’s biosynthesis and, according to this observation, *Trichoderma* IAA contributes to exogenous auxin-stimulated ET biosynthesis via ACC synthase, which in turn triggers an increase in abscisic acid biosynthesis. Depending on the timing and outcome of *Trichoderma* stimuli, phytohormone homeostasis will control plant development and immune responses.

The groundbreaking work of [12] showed that *Trichoderma* genes can be effectively expressed in plants to bestow advantageous traits, primarily in the control of plant ailments. The *T. harzianum* endochitinase gene chit42 was found to be highly expressed in a variety of plant tissues in that study, with no discernible impact on the growth or development of the plants.

Selected transgenic lines of potato and tobacco were either entirely resistant to the soil-borne disease *Rhizoctonia solani* and the leaf pathogens *Alternaria alternata*, *Alternaria solani*, and *B. cinerea*. Chit42 expression dramatically boosted the resilience of broccoli to attack by *Alternaria brassicicola* and decreased plant growth while increasing resistance to *Venturia inaequalis* in apple.



Genes responsible for *Trichoderma* activity

The expression of chit42 in lemon enhanced resistance to *Phoma tracheiphila* and *B. cinerea*, a significant correlation between resistance and transgene expression being observed, with an upregulation of ROS and JA/ET-responsive genes. The homologous chit42 gene from *T. virens* was able to enhance resistance against *R. solani* when it was expressed in rice [13]. Other *Trichoderma* chitinase genes have been used to generate transgenic plants resistant to fungal diseases: (i) tobacco cell cultures expressing the *T. harzianum* endochitinase chit40 gene released the enzyme into the medium and were able to inhibit the conidial germination of the post-harvest pathogen *Penicillium digitatum*; (ii) transgenic cotton plants expressing the *T. virens* endochitinase gene Tv-ech1 showed significant resistance to *A. alternata* and *R. solani* and a rapid and greater induction of ROS, followed by modulation of the expression of several defence-related genes and the induction of the terpenoid pathway; (iii) the expression of the endochitinase chit36 gene of *T. harzianum* in carrot significantly enhanced tolerance to *A. radicina* and *B. cinerea*. Abiotic stress tolerance in plants is accompanied by growth inhibition after overexpression of heat-shock genes of plant origin. Thus, an important biotechnological advantage has been the expression of a *T. harzianum* hsp70 gene in Arabidopsis to induce resistance to high temperatures, high salinity and drought without loss of vigour and growth or developmental alterationsprobably due to the heterologous nature of the transgene. Recent examples of biotechnological solutions from *Trichoderma* are the *T. harzianum* Thkel1 gene, encoding a kelch-repeat protein involved in the modulation of glucosidase activity that enhanced seed germination and plant tolerance to salt and osmotic stresses when it was expressed in

Arabidopsis [14], and transgenic tobacco plants expressing a *T. virens* glutathione transferase to improve their remediation and xenobiotic degradation potential [15].

Conclusion:

Genomes of *Trichoderma* species have identified mycotrophy and mycoparasitism as their ancestors' modes of subsistence. *Trichoderma* strains that have established themselves as intercellular root colonizers, they developed in the rhizosphere of plants. They thus promote plant development and pathogen defenses. *Trichoderma*, like other advantageous microorganisms, induces ISR via JA/ET-dependent pathways and initiates priming responses in the plant. However, depending on the *Trichoderma* strains and concentrations used, the plant material, the developmental stage of the plant, and the timing of the interaction, the expression of defense-related genes of the JA/ET and/or SA pathways may overlap. The phytohormones ET and IAA, which play a part in connecting plant development and defense mechanisms, are also produced by *Trichoderma*. Plants that have *Trichoderma* genes expressed are more resistant to environmental stressors and are less likely to contract plant diseases. The experimental data discussed here suggests that *Trichoderma*-plant interactions share traits with other advantageous microbe relationships but also exhibit unique traits because of *Trichoderma*'s unique lifestyle. However, additional research is required to understand the signaling transduction pathways involved in defense and development that emerge from interactions between *Trichoderma* plants and pathogens or other types of abiotic stress.

References:

1. Van Wees, S. C. M., van der Ent, S. and Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11, 443–448.
2. Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D. A., Druzhinina, I. S., Thon, M., Zeilinger, S., Casas-Flores, S., Horwitz, B. A. and other authors (2011). Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol 12, R40.
3. Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., Mukherjee, P. K., Zeilinger, S., Grigoriev, I. V. and Kubicek, C. P. (2011). *Trichoderma*: the genomics of opportunistic success. Nat Rev Microbiol 9, 749–759.
4. Shoresh, M., Harman, G. E. and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. Annu Rev Phytopathol 48, 21–43.
5. Ramírez-Valdespino CA, Casas-Flores S and Olmedo-Monfil V (2019) *Trichoderma* as a Model to Study Effector-Like Molecules. Front. Microbiol. 10:1030.
6. Viterbo, A. and Chet, I. (2006). TasHyd1, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum*, is involved in plant root colonization. Mol Plant Pathol 7, 249–258.

7. Rotblat, B., Enshell-Seijffers, D., Gershoni, J. M., Schuster, S. and Avni, A. (2002). Identification of an essential component of the elicitation active site of the EIX protein elicitor. Plant J 32, 1049–1055.
8. Brotman, Y., Briff, E., Viterbo, A. and Chet, I. (2008). Role of swollenin, an expansin-like protein from *Trichoderma*, in plant root colonization. Plant Physiol 147, 779–789.
9. Engelberth, J., Koch, T., Schuler, G., Bachmann, N., Rechtenbach, J. and Boland, W. (2001). Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiol 125, 369–377.
10. Luo, Y., Zhang, D. D., Dong, X. W., Zhao, P. B., Chen, L. L., Song, X. Y., Wang, X. J., Chen, X. L., Shi, M. and Zhang, Y. Z. (2010). Antimicrobial peptaibols induce defense responses and systemic resistance in tobacco against tobacco mosaic virus. FEMS Microbiol Lett 313, 120–126.
11. Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., Woo, S. L. and Lorito, M. (2008). A novel role for *Trichoderma* secondary metabolites in the interactions with plants. Physiol Mol Plant Pathol 72, 80–86.
12. Lorito, M., Woo, S. L., Garcı´a, I., Colucci, G., Harman, G. E., PintorToro, J. A., Filippone, E., Muccifora, S., Lawrence, C. B. and other authors (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA 95, 7860–7865.
13. Shah, J. M., Raghupathy, V. and Veluthambi, K. (2009). Enhanced sheath blight resistance in transgenic rice expressing an endochitinase gene from *Trichoderma* virens. Biotechnol Lett 31, 239–244.
14. Hermosa, R., Botella, L., Keck, E., Jime´ nez, J. A., MonteroBarrientos, M., Arbona, V., Go´ mez-Cadenas, A., Monte, E. and Nicola´ s, C. (2011). The overexpression in Arabidopsis thaliana of a *Trichoderma* harzianum gene that modulates glucosidase activity, and enhances tolerance to salt and osmotic stresses. J Plant Physiol 168, 1295–1302.
15. Dixit, P., Mukherjee, P. K., Sherkhane, P. D., Kale, S. P. and Eapen, S. (2011). Enhanced tolerance and remediation of anthracene by transgenic tobacco plants expressing a fungal glutathione transferase gene. J Hazard Mater 192, 270–276.

METHOD FOR DETECTION OF PLANT PATHOGENS- LAMP (LOOP MEDIATED ISOTHERMAL AMPLIFICATION)

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Abstract:

Loop mediated isothermal amplification (LAMP) is a innovative gene amplification technique emerging as a simple, rapid, diagnostic tool for early detection and identification of plant pathogens. Amplification involves four set specially designed primers which identify six distinct sequences of a target gene. The sequences and sizes of the primers were chosen such that their Tm values fell between 60^0C and 65^0C. DNA polymerase used this method has strand displacement activity. Amplification of target gene can complete in a simple water bath under isothermal condition. Amplified products can be detected by agarose gel electrophoresis or real-time monitoring in an inexpensive turbidimeter or simply by naked eye as turbidity changes upon amplication. LAMP can amplify large amount of DNA from few copies within 30 minutes with great specificity and higher sensitivity. Presence of target gene indicated by presence of amplified product as it is highly specific. Both amplication and detection of gene of interest can be completed in a single step and in a single tube. This method is cost effective as it does not require special reagents or sophisticated equipment like thermal cycler. Considering the advantages of rapid amplification, simple operation and easy detection, LAMP has potential applications for detection of plant pathogens without requiring thermal cycler.

Introduction:

LAMP which stands for Loop-mediated Isothermal Amplification is a simple, rapid, specific and cost-effective nucleic acid amplification method solely developed by Eiken Chemical Co., Ltd. It is characterized by the use of 4 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction. Amplification and detection of gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). It provides high amplification efficiency, with DNA being amplified 10^9-10^{10} times in 15-60 minutes. Because of its high specificity, the presence of amplified product can indicate the presence of target gene.

Characteristics of LAMP

- There is no need for a step to denature double stranded into a single stranded form.
- The whole amplification reaction takes place continuously under isothermal conditions

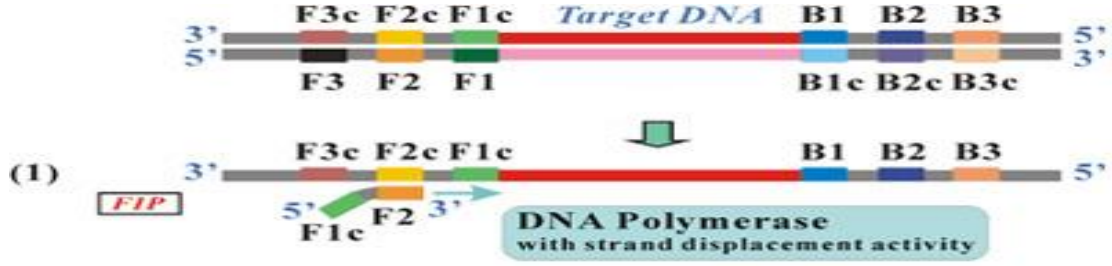
- The amplification efficiency is extremely high
- By designing 4 primers to recognize 6 distinct regions, the LAMP method is able to specifically amplify the target gene.
- The total cost can be reduced, as LAMP does not require special reagents or sophisticated equipments
- The amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand
- Amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase.

Principle of LAMP

When the target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60-65°C, the following reaction steps proceed.

Step 1

As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA. As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded.

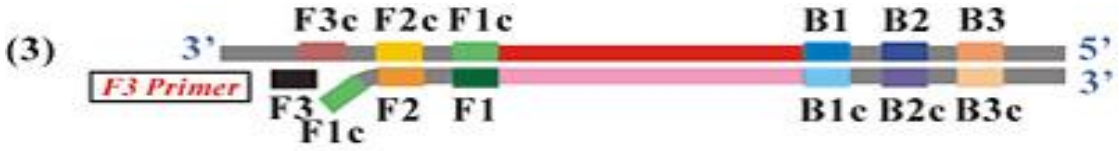


Step 2

Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP.

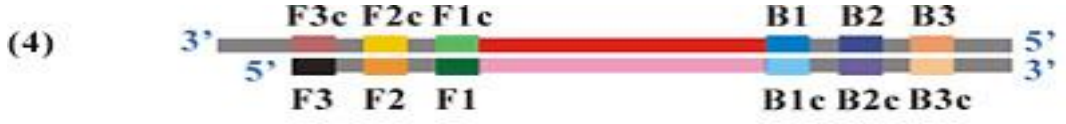
Step 3

The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand.



Step 4

A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand.



The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions.

Step 5

Step 6

This single strand DNA in Step (5) serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in Step (5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer.

Step 7

Double stranded DNA is produced through the processes described in Step (6).

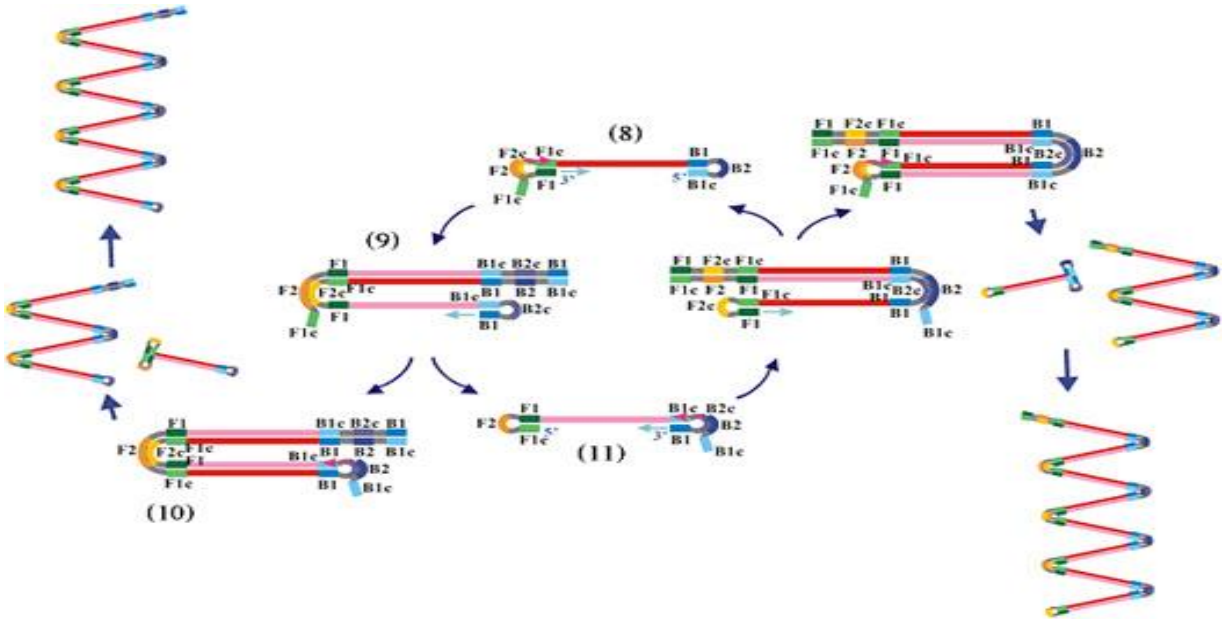
Step 8

The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting

structure for the amplification cycle in the LAMP method (LAMP cycling). The above process can be understood as producing the starting structure for LAMP cycling.

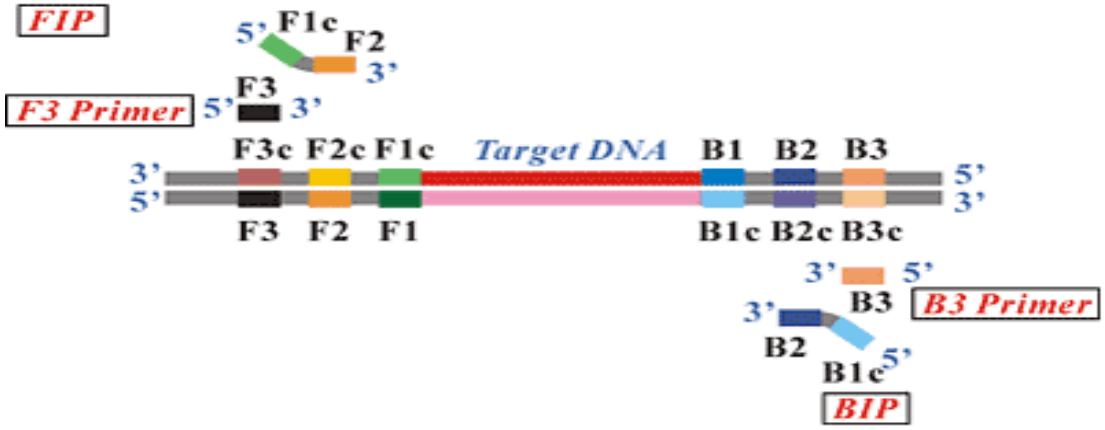
Basic principle cycling amplication

A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (Step (9)). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (Step (11)). This structure is the 'turn over' structure of the structure formed in Step (8). Similar to the Steps from (8) to (11), structure in Step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to Steps (9) and (10) as well as the same structure as Step (8) are produced. With the structure produced in Step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.



Design of primers

Design 4 types of primers (described in detail below) based on the following 6 distinct regions of the target gene: the F3c, F2c and F1c regions at the 3' side and the B1, B2 and B3 regions at the 5' side.



Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end. Forward Outer Primer consists of the F3 region that is complementary to the F3c region Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. Backward Outer Primer consists of the B3 region that is complementary to the B3c region.

Main points

Proper primer design is crucial for performing LAMP amplification. The above primer regions can be determined by using the PrimerExplore (a special software to design LAMP primers) after considering the base composition, GC contents and the formation of secondary structures. Tm value can be obtained by Nearest Neighbor method. The following is the main points of primer design

Distance between primer regions

The distance between 5' end of F2 and B2 is considered to be 120-180bp, and the distance between F2 and F3 as well as B2 and B3 is 0-20bp.

The distance for loop forming regions (5' of F2 to 3' of F1, 5' of B2 to 3' of B1) is 40-60bp.

Tm value for primer regions

About 60-65°C in the case of GC rich and Normal, about 55-60°C for AT rich

The stability of primer end

The dG calculated on 6bp from the following end regions should be less than -4kcal/mol, 5' end of F1c/B1c and 3' end of F2/B2 as well as F3/B3.

GC contents

About 50-60% in the case of GC rich and Normal, about 40-50% for AT rich.

Secondary structure

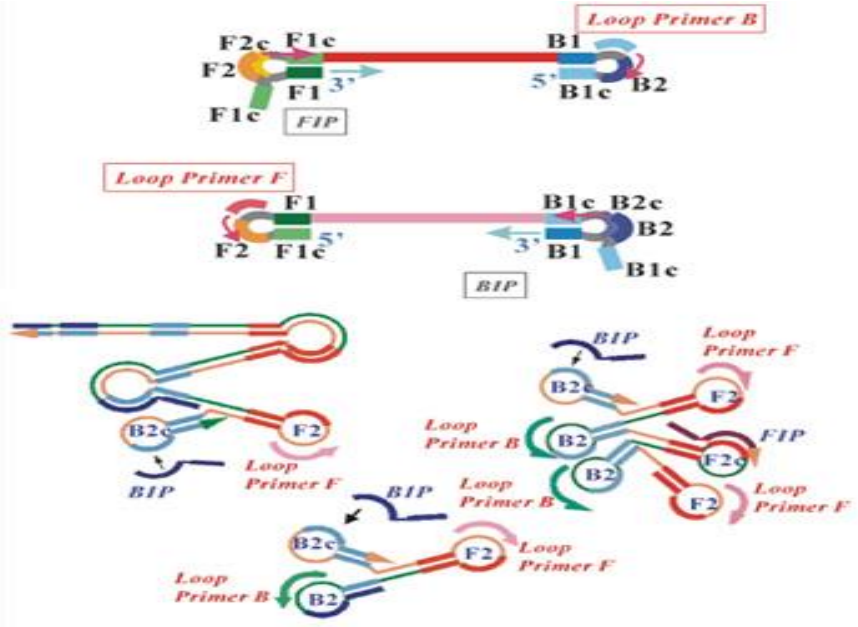
Primers should be designed so as not to easily form secondary structures. 3' end sequence should not be AT rich or complementary to other primers.

Others

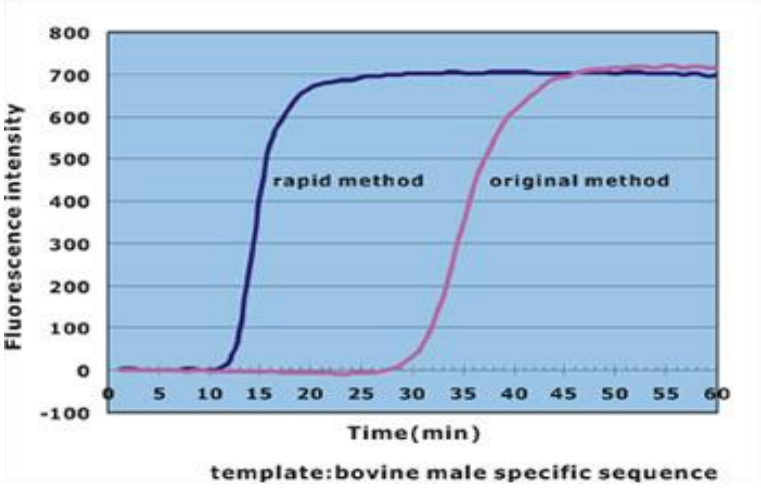
If the restriction enzyme sites exist on the target sequence, except the primer regions, they can be used to confirm the amplified products

Principle of Loop Primers

The Loop Primers (either Loop Primer B or Loop Primer F), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis for the LAMP method. An example is shown in the figure where there is an amplified product containing six loops. In the original LAMP method, four of these loops would not be used, but through the use of Loop Primers, all the single stranded loops can be used as starting points for DNA synthesis.



The investigation on how Loop Primers affect amplification time (original method: no Loop Primer; rapid method: with Loop Primers) shows that the time required for amplification with Loop Primers is one-third to one-half of that without Loop Primer. With the use of Loop Primers, amplification can be achieved within 30 minutes.



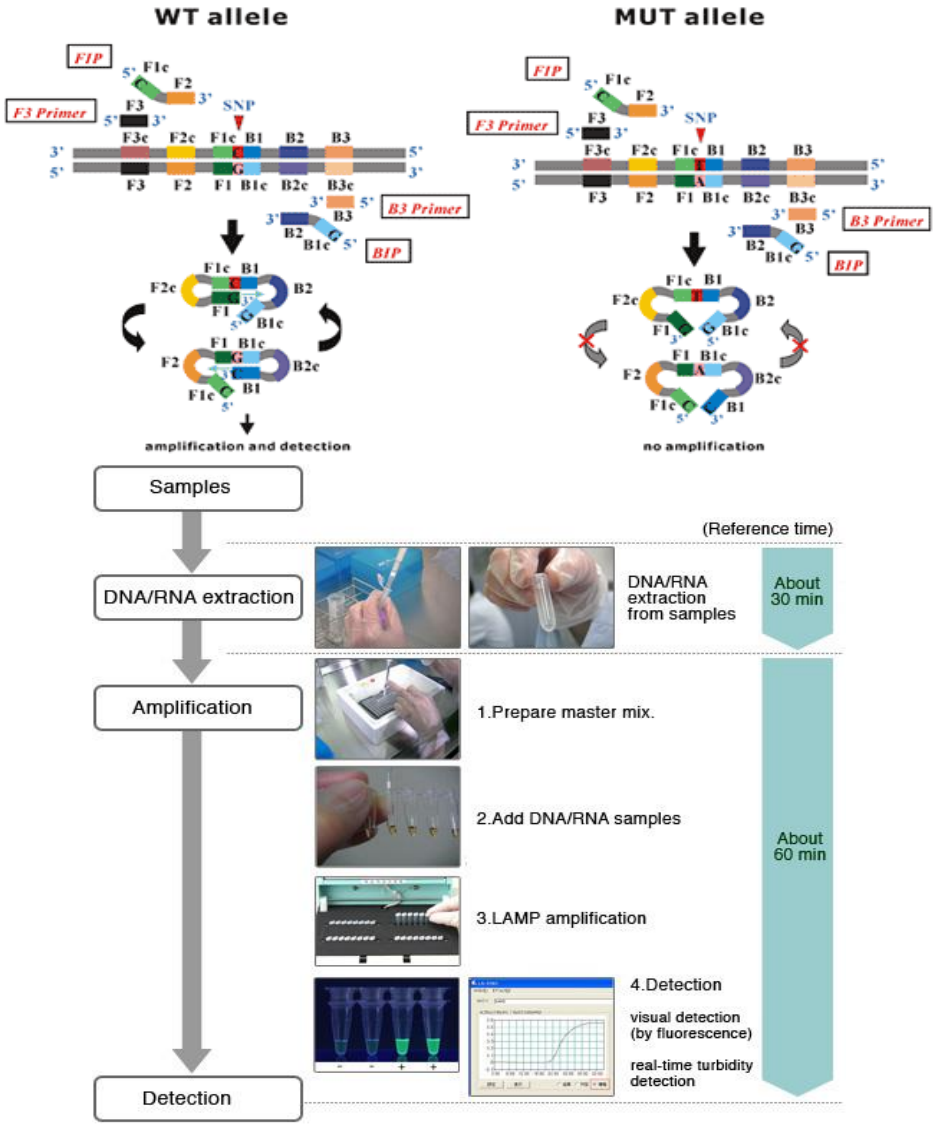
Reverse Transcription-Loop-mediated Isothermal Amplification

RT-LAMP (Reverse Transcription-Loop-mediated Isothermal Amplification) method can synthesize cDNA from template RNA and apply LAMP technology to amplify and detect them. As the template is RNA sample, in addition to the reagents of DNA amplification (primers, DNA

polymerase with strand displacement activity, substrates, etc.), reverse transcriptase is added to the reaction mixture. After mixing and incubating at a constant temperature between 60-65°C, amplification and detection can be carried out in a single step. Use RNA extraction kit to extract RNA from the samples, and then prepare the sample solution. Mix the sample solution and the reaction solution and incubate them at a constant temperature between 60-65°C. As shown in the following figure (1), BIP anneals to the template RNA, and with the activity of reverse transcriptase, cDNA is synthesized.

SNP typing

The figures below show the basic principles of the process when using Wild Type (WT) Primers. The FIP and BIP are designed to contain a SNP nucleotide (in this case of Wild Type allele) at 5' end, respectively. Using the WT primers, when the target gene is the WT allele, DNA synthesis from dumbbell-like starting structure proceeds and the LAMP amplification cycling continues. In contrast, when the target gene is the Mutant (MUT) allele, no DNA synthesis proceeds from dumbbell-like structure and the LAMP amplification cycling dose not occur. Even if DNA synthesis proceeds in one step due to miscopy, the amplification reaction is either halted in other steps or is delayed since repetition of this reaction.



Conclusion

LAMP serves as alternative amplication method to detect plant pathogens without thermal cycler. It is very rapid highly sensitive, highly specific, cost effective nucleic acid amplication method. Popularization and commercialization of technique is needed. Loop-mediated isothermal amplification (LAMP) is a molecular biology technique used for the rapid and sensitive detection of specific DNA or RNA sequences. It is an isothermal amplification method, meaning that it does not require a thermal cycling process like polymerase chain reaction (PCR). In LAMP, a DNA or RNA template is mixed with a set of specially designed primers, including two outer primers and two inner primers. The primers are designed to recognize and bind to specific regions within the target nucleic acid sequence. The primers initiate strand displacement and DNA synthesis, resulting in the generation of large amount of amplified DNA.

References:

1. https://applbiolchem.springeropen.com/articles/10.1007/s13765-017-0267-y#:~:text=LAMP%20is%20a%20rapid%20and,activity%20of%20Bst%20DNA%20polymerase.
2. https://www.frontiersin.org/articles/10.3389/fpls.2022.857673/full
3. https://apsjournals.apsnet.org/doi/10.1094/PDIS-05-18-0858-RE

MECHANISM OF *BACILLUS STRAINS* AS DEFENCES AGAINST BACTERIAL LEAF BLIGHT OF RICE

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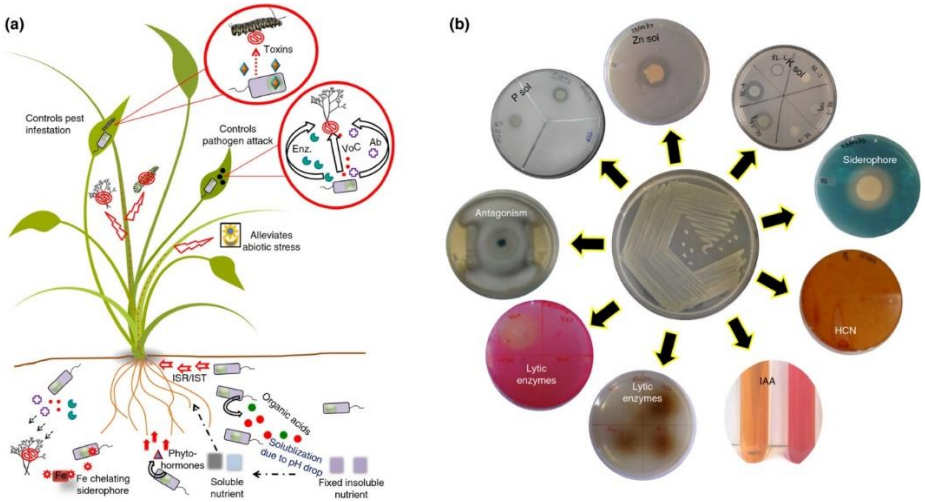
Abstract:

Bacillus strains are extensively researched for their beneficial effects on plant growth and biological control of plant diseases and pests, little is known about their underlying mechanisms. *B. subtilis* A15, which is associated with rice seeds, along with the rhizobacterial strains *B. amyloliquefaciens* D29 and *B. methylotrophicus* H8 have all been evaluated for their ability to control and mount defences against bacterial leaf blight (BLB), which is brought on by *Xanthomonas oryzae* pv. *oryzae.* According to the findings, each of the three strains was highly capable of forming biofilms. *X. oryzae* growth and development of biofilms were significantly inhibited by the culture filtrates of each strain, and transmission electron microscopy images revealed changes in bacterial cell morphology, including cell swelling and severe cell wall alterations. According to PCR results, all three strains have the antimicrobial-associated genes necessary for the manufacture of the antibiotics bacillomycin, fengycin, iturin, and surfactin. Following real-time qPCR testing, it was discovered that D29, H8, and A15 had elevated expression of the fenD and srfAA genes as well as the fenD and ituC genes during their in vitro contact with *X. oryzae*. It implies that the three strains' antibacterial defence mechanisms may be at least somewhat linked to their capacity to secrete the respective lipopeptides. It is interesting to note that the three strains applied under greenhouse circumstances were successful in suppressing the Rice BLB. This was accomplished by activating systemic resistance caused by the increased activities of defence-related enzymes.

Introduction:

One of the commercially significant farmed crops is rice (*Oryza sativa* L.). The greatest obstacles to the production of rice are thought to be losses brought on by illnesses and pests. One of the most serious diseases of rice is bacterial leaf blight, which is brought on by the *Xanthomonas oryzae pv. oryzae* bacterium. Adult plants show symptoms as water-soaked yellowish stripes on leaf blades or beginning at leaf tips. These stripes grow longer and wider and eventually kill the infected leaves. Plants that are infected develop sterile, empty panicles, and finally wilt and die. The primary way to maintain rice yield is to reduce disease from year to year and the frequency of outbreaks. For the purpose of promoting development, seed emergence, and disease management in rice, various potential antagonists have historically been identified from the rhizosphere and commercialized [1]. Numerous Bacillus species are known to be antagonistic microorganisms that produce a variety of antimicrobial chemicals with different structural compositions that inhibit the growth of plant diseases. According to reports, different species of Bacillus have the ability to colonize plant roots and trigger a variety of resistance

responses in both the plant's underground and above-ground portions. Bacillus strains exhibit a variety of cytological characteristics, including the production of stress-resistant endospores, the release of peptide antibiotics, multilayered cell walls, and peptide signal molecules, all of which are important for the survival of Bacillus strains in harsh environments. Antibiotic production, the manufacture of extracellular enzymes that compete for rhizosphere niches and hydrolyse bacterial and fungal cell walls, and the induction of systemic resistance are just a few of the antimicrobial-related mechanisms that have been put forth [2]. New insights into the genetic basis of interactions between phytopathogens, biocontrol agents, and plants have been made possible by molecular and biochemical methods. For instance, *B. subtilis* plays complementary roles in the induction and enhanced expression of three genes important in controlling plant cell development. The genome sequence analysis of the biocontrol strains *B. amyloliquefaciens* FZB42 [3] and *B. subtilis* [4] found that, respectively, 8.5% and 4% of the genomes are dedicated to the secondary metabolism. Due to their functions in the restriction of microbial development, the lipopeptides surfactin, fengycin, and iturin as well as the polyketide antibiotics bacillaene, difficidin, and macrolactin have previously received the majority of attention [3]. Plants pretreated with biocontrol agents showed increased polyphenol oxidase (PPO) and phenyl ammonia lyase (PAL) activity. Plants challenged with pathogen inoculation resulted in the accumulation of phenol, and hosts were subsequently protected by inducing systemic resistance (ISR). According to reports, biocontrol agent-mediated ISR increased the production of antioxidant enzymes such peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), which protected the plants from a variety of diseases. Although numerous biocontrol agents have been identified for rice plants, little is known about how these compounds interact with bacteria.



[5]

About bacillus strain- production and their multiplication

LB broth was used to grow the bacterial strains, and an overnight culture of each strain was diluted to an OD600 of 0.3, or roughly 107 cfu/ml. Then, in a 96-well plastic microtitre plate, 5 ll of bacterial suspension was mixed to 195 ll of pure LB broth. As a negative control, wells filled with only pure LB broth were measured. For 24 hours, the plate was kept at 37 °C. The cultures were then gently taken out of the wells. The remaining cells and matrices were

stained with 150 ll of 1% crystal violet solution at 37°C for 30 min after each well had been lightly rinsed twice with double-distilled water (ddWater). The CV adhered to the biofilm was washed twice with ddWater before being dissolved in 150 cc of 33% acetic acid. Using 96-well microtitre culture plates, the efficacy of each strain and SA to prevent *X. oryzae pv. oryzae* from forming biofilms was evaluated. In 96-well plates, *X. oryzae pv. oryzae* was cultivated in LB broth (OD600 = 0.8; 108 cfu/ml) at 28°C for 12 hours before 5 ll of the pathogen culture was added and the plate kept at 30°C for 24 hours. Then each well received 100 ll of culture filtrate (70%) for the tested strains. In the six wells designated for the negative control, just LB broth was used [6]. The plate was then incubated for a further hour at 30°C. Following that, all non-attached cells were eliminated by removing the culture medium, gently washing the plate four times with ddWater, and drying the plate for one hour. The connected biofilm sample was dyed for 25–30 minutes at room temperature (TM) using 1% CV. The stained solution was solubilized with 150 ml of 33% acetic acid after incubation, and its absorbance was gauged at 570 nm [6]. Each Bacillus strain's culture filtrate was made as previously described, and 500 ll *X. oryzae* culture (108 cfu/ml) was then added to a sterile 10 ml tube containing LB broth (3 ml) and culture-free supernatant (70%) and incubated for 4 hours at 30°C on a rotary shaker (160 rpm). Each sample was then prepared for TEM using a method that was previously reported [7]. Each Bacillus strain's culture filtrate was made as previously described, and 500 ll *X. oryzae* culture (108 cfu/ml) was then added to a sterile 10 ml tube containing LB broth (3 ml) and culture-free supernatant (70%) and incubated for 4 hours at 30°C on a rotary shaker (160 rpm). Each sample was then prepared for TEM using a method that was previously reported [8]. Rice leaf blight caused by bacteria was investigated in vivo for biological control using suspensions of the strains A15, D29, and H8. Rice seeds were sterilized for two minutes in a solution of 2% sodium hypochlorite before being washed five times with sterile distilled water. Seeds were treated by immersing them in a 108 cfu/ml bacterial suspension for eight hours. As a control, seeds were submerged in sterile saline solution. The seeds were subsequently germinated on Petri dishes for 3 days at 28°C in a growth chamber. The germinated seeds were planted in pots with sterilized peaty soil and kept in a greenhouse with 28°C daytime temperatures, 25° night time temperatures, and relative humidity levels between 70 and 90 percent [7]. According to the instructions provided by the manufacturer, the TIANamp Bacteria DNA Kit (Tiangen Biotech co., Ltd., Beijing, China) was used to extract the genomic DNA of each Bacillus strain. The presence of biosynthesis genes in the Bacillus strains (A15, D29, and H8) was determined using four AMP biosynthetic genes (srfAA, surfactin; bacA, bacilysin; fenD, fengycin; and ituC, iturin). As a positive control, Bacillus strain 4812 was employed, while sterile distilled water devoid of DNA served as a negative control [9]. A PCR assay kit from TaKaRa, Dalian, China, was used in accordance with the manufacturer's instructions. The reaction mixture, which contained mix 2 (10 ll), primers (0.5 ll each), and templates (1 ll each), was carried out in a total volume of 20 ll. Using real-time quantitative PCR, the expression changes in genes linked to the antibacterial activity of Bacillus strains were investigated during their in vitro contact with *X. oryzae.* [10] created four realtime PCR primers based on the coding regions of the srfAA, bacA,

fenD, and ituC genes. Using the dual culture approach, the three Bacillus strains were grown on LBA plates (either with or without the *X. oryzae*).

Detection of growth promoting and bio control determinants

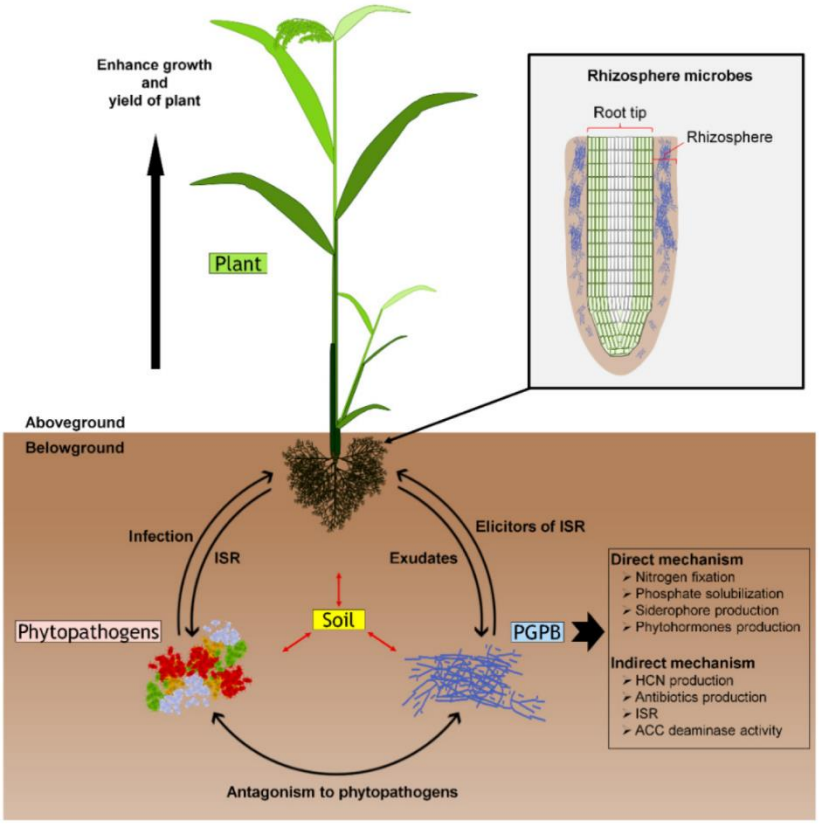
Acetylene reduction assay was used to calculate the amount of nitrogen fixed by bacteria. On Pikovskaya, the bacterium's solubilization of phosphate was measured. Insoluble tricalcium phosphate was added to agar medium, and the amount was measured using the phospho-molybdate blue color method. The method was used to quantify the generated Indole Acetic Acid (IAA). In vials containing 3 L of 1-aminocyclopropane-1-carboxylic acid (ACC) (0.5 M) as the only N source in 5 mL of DF salt minimum medium), the activity of the enzyme was measured. Scientist observed the production of siderophores on universal chrome azurol "S" (CAS) agar media. Certain method was used to detect the production of HCN. On skim milk agar [11] and chitin agar media, protease and chitinase activity were found. On minimal medium supplemented with glucan source, i.e. Lamimarin containing 0.5% yeast extract, glucanolytic activity was found. On nutritional agar with 2% starch added, starch hydrolyzing capacity was found. Each growth-promoting and biocontrol determinant evaluated had three biological replicates.

Studies of rhizosphere colonization

Viable count was employed to track changes in the BRp3 population linked to the rice variety Super Basmati. The colonies of the competing strain had a peculiar morphology. LB agar plates produced green pigments, which were employed as a monitoring tool. In addition, the pattern of antibiotic resistance, antagonistic behavior toward Xoo strains, generation of IAA and siderophores, and P solubilization were used to further identify the implanted bacterium. Each replicate field plot had three root and shoot samples taken 14, 21, 40, and 60 days following treatment. Samples of roots and shoots underwent a brief sterile distilled water wash. The shoot pieces were crushed in 10 mL of sterile phosphate buffer. Super Basmati rice seeds that had been surface sterilized were planted in small plastic pots with 50 g of air-dried, sieved-sterilized sand as part of an experiment. There were non-vaccinated pots treated separately as the control. (109 CFU mL1) of BRp3 was seed injected. During the rice-growing season, every pot was maintained in a net house. After the seeds germinated, plants were harvested after 21 days. RhITC conjugate was used to prevent nonspecific stain adsorption. The roots were stained using a particular FA [12]. The fluorescent bacteria were observed using a confocal laser scanning microscope (Olympus FV1000, Japan) equipped with an Argonion laser and FV10-ASW 1.7 imaging software at 488 and 525 nm for FITC absorption and emission, respectively.

In modern agriculture, where eco-friendly and sustainable methods are expanding and have become more prevalent, plant-associated beneficial bacteria are significant growth boosters or biocontrol agents greater acceptance than ever. In India rice is farmed over a huge region with the use of numerous chemical fertilizers and pesticides. This research was done to see whether rhizobacterial inoculum could help rice grow and fight disease. X. oryzae isolates were identified as the BLB causative pathogens by their pathogenicity to rice. Due of their aggressive demeanor, Xoo1 and Xoo2 were used for more research. The method for testing the rhizobacterial isolates

(512) for Xoo growth inhibition resulted in the choice of BRp3 for additional research. Only LB medium was employed in a prior work to identify antagonistic bacteria, and only a small percentage of the isolated bacterial community included any antagonistic bacteria.



Conclusion:

This book chapter has been devoted to the bidirectional interaction between helpful soil- and plant-associated bacteria and their host plant provided a clear explanation of how some bacilli might inhibit plant diseases and stimulate plant development. Here, different Bacillus strains were examined in order to choose the right Bacillus strains with the greatest formulation performance against the *Xanthomonas oryzae* caused BLB disease in rice. The three strains of *B. subtilis* A15, *B. amyloliquefaciens* D29, and *B. methylotrophicus* culture filtrates that were investigated considerably suppressed the growth of *X. oryzae* H8, particularly the metabolites produced. The Bacillus genus produces a wide range of bioactive molecules that inhibit the growth of plant pathogens, and because surfactants have a surfactant nature, antimicrobial compounds of the surfactin, iturin, and fengycin families have well-recognized potential to be applied. Obtainable reports on the ability of Bacillus species to form biofilm have recommended that the endophytic colonization and biofilm formation improve the bacterial ability to control phytopathogens. On the capacity of Bacillus species to prevent pathogen biofilm development, numerous studies have been reported. For instance, *B. cereus*, *B. thuringiensis*, and *B. mycoides* all possess the capacity to create acyl-L-homoserine lactone (AHL)-inactivating (quorum quenching) biofilm-associated enzymes. Due to the presence of an AHL lactonase (aiiA) expressing gene in *B. amyloliquefaciens*, the quorum quenching feature is also a possible strategy to prevent the proliferation of plant pathogens. Images taken using transmission electron

microscopy showed several morphological changes that occurred in *X. oryzae* after the addition of *B. amyloliquefaciens* cell filtrate. The outer layer of the *X. oryzae* cell was noticeably disorganized, as shown by the wrinkled surfaces and inflated cell structures, which validates the postulated lipopeptide- and antibiotic-associated mechanisms in cell disruption.

References:

1. Yang JH, Liu HX, Zhu GM, Pan YL, Xu LP, Guo JH. (2008). Diversity analysis of antagonists from rice-associated bacteria and their application in biocontrol of rice diseases. J Appl Microbiol 104:91–104.
2. Waewthongrak W, Leelasuphakul W, McCollum G. (2014). Cyclic lipopeptides from Bacillus subtilis ABS–S14 elicit defense-related gene expression in citrus fruit. PLoS ONE 9:e109386.
3. Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O. (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium Bacillus amyloliquefaciens FZB42. Nat Biotechnol 25:1007–1014.
4. Earl AM, Losick R, Kolter R. (2008). Ecology and genomics of Bacillus subtilis. Trends Microbiol 16:269–275.
5. Antonio Camillo de Carvalho Marco, Eustáquio de Sá Marco, Tiago da Silva Campos Daniela, Paulo Machado Alexandre, Freitas Chagas Junior Aloisio (2023). *Bacillus subtilis* UFMT-Pant001 as a plant growth promoter in soybean in a greenhouse, African Journal of Agricultural Research, 10.5897/AJAR2022.16232, 19, 2, (161-169).
6. Elshakh, A. S. A., Anjum, S. I., Qiu, W., Almoneafy, A. A., Li, W., Yang, Z., Xie, G.-L. (2016). Controlling and Defence-related Mechanisms ofBacillusStrains Against Bacterial Leaf Blight of Rice. Journal of Phytopathology, 164(7-8), 534–546.
7. Helander I, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol 71:235–244.
8. Kakar KU, Duan YP, Nawaz Z, Sun G, Almoneafy AA, Hassan MA, Elshakh A, Li B, Xie GL. (2014). A novel rhizobacterium Bk7 for biological control of brown sheath rot of rice caused by Pseudomonas fuscovaginae and its mode of action. Eur J Plant Pathol 138:819–834.
9. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. (2012). Primer3 – new capabilities and interfaces. Nucleic Acids Res 40:e115.
10. Denizci, D., Kazan, E. C. A., and Erarslan, A. A. (2004). Newly isolated Bacillus clausii GMBAE42, an alkaline protease producer capable to grow under highly-alkaline conditions. J. Appl. Microbiol. 96, 320–327.
11. Yasmin, S., Hafeez, F. Y., and Rasul, G. (2014). Evaluation of Pseudomonas aeruginosa for biocontrol of cotton seedling disease caused by Fusarium oxyporum. Biocontrol Sci. Technol. 24, 1227–1242.

IN VITRO ANTIFUNGAL ACTIVITY OF*PARTHENIUM HISTEROPHORUS* AGAINST *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* CAUSING PANAMA WILT OF BANANA

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Abstract:

Fusarium oxysporum f.sp.*cubense* (Panama Wilt of Banana) is an important disease that causes wilt disease in Banana crop in India and all over world. Management through chemical fungicides causes resistance in pathogen and causes damage to non targeted organisms. Congress grass, *Parthenium hysterophorus* L., of the family Asteraceae [tribe: Heliantheae], is notorious weed distributed all over. Therefore, in present investigation in vitro biological control of *Fusarium oxysporum* f. sp. *cubense* was conducted by using plant extract from stem of *Parthenium histerophorus*. Potential of *Parthenium histerophorus* was tested against the *Fusarium oxysporum* f. sp*cubense* by well agar method.

Keywords: *Fusarium oxysporum* f.sp.*cubense*, Panama wilt, *Parthenium histerophorus* .

Introduction:

Biological control of plant pathogens is preferred over the hazardous chemical based products. The plants serve as food, medicines, raw product for industry and antifungal sources. The plant extracts serve as ecofriendly and chief antifungal source. Banana (*Musa* spp.) is an important source of human nutrition, providing food and income to millions of people in the world. Banana is cultivated in Asia, Australia, Africa, North and South America [Rangaswami, 2002]. The banana is attacked by many fungal pathogens. Among them Fusarium wilt of banana is most destructive disease (Sebasigari, 1988; Stover, 1962). Panama wilt is caused by *Fusarium oxysporum*f.sp. *cubense* (Smith, 1910; Snyder and Hansen, 1940). In the present investigation solvent and aqueousstem extract of *Parthenium histerophorus* were tested for their antifungal activity against *Fusarium oxysporium* f. sp. *cubense*.

Material and Methods:

Crudestem extract preparation forthis study, 20.0 g dry powdered material was extracted with 200 ml of alcohol and aqueous extracts in cold maceration method using aspirated bottle and the extract thus obtained was dried in vacuum.

Table 1: Effect of *Parthenium histerophorus* stem extract on radial growth (mm) benomyl sensitive isolate of *Fusarium oxysporium*f. sp. *cubense.*

Concentration %	Plant extract	Days			
		2	4	6	8
10	Alcoholic	09.66	12.66	15.33	19.33
	Aqueous	24.33	55.00	61.66	70.33
25	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	19.66	47.33	54.00	64.33
50	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	17.66	38.66	45.66	53.33
75	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	14.66	31.66	37.66	44.66
100	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	9.66	21.00	32.33	37.33
	Control	21.00	42.33	62.00	80.00

Significance by two way analysis of variance [ANOVA]

	Alcoholic	Aqueous
Between days F	1.40	56.90
Between concentrations F	16.39	15.43
C.D. at P=0.05	12.50	6.90
C.D. at P=0.01	17.31	9.55

Table 2: Effect of *Parthenium histerophorus* stem extract on radial growth (mm) benomyl resistant isolate of *Fusarium oxysporium* f. sp. *cubense*

Concentration %	Plant extract	Days			
		2	4	6	8
10	Alcoholic	09.33	10.66	11.66	13.66
	Aqueous	20.33	41.33	55.33	64.00
25	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	17.00	39.33	51.33	58.33
50	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	12.66	31.33	42.33	52.33
75	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	11.33	21.66	29.00	38.00
100	Alcoholic	00.00	00.00	00.00	00.00
	Aqueous	00.00	16.66	26.33	32.66
	Control	25.00	45.33	64.33	80.00

Significance by two way analysis of variance [ANOVA]

	Alcoholic	Aqueous
Between days F	1.18	86.09
Between concentrations F	20.02	32.67
C.D. at P=0.05	11.17	5.55
C.D. at P=0.01	16.31	7.69

Results and Discussion:

Alcoholic and aqueous stem extract of *Parthenium histerophorus* was evaluated against benomyl sensitive and resistant isolates of *Fusarium oxysporium* f. sp. *cubense* in vitro. Alcoholic stem extract had completely inhibited the mycelial growth of both sensitive and resistant isolates of *Fusarium oxysporium* f. sp. *cubense* at 25 % concentration. However, the tested aqueous stem extract was significantly less effective. Alcoholic plant extracts showed high inhibition of pathogen due to solubility of the active compound (s) required for antifungal activity.

References:

1. Rangaswami, G. and Mahadevan, A. (2002).: Diseases of Crop plants in India. 4th ed. Pretice Hall India.
2. Sebasigari K. and Stover, R. H. (1988).: Banana diseases and pests in East Africa. Report of a Survey in November, 1987, INIBAP, Montpellier, France, pp.15–17.
3. Smith E. F. (1910). A Cuban banana disease. (Abstract.) Science, 31: 754-755.
4. Snyder, W. C. and Hansen, H. N. (1940). The species concept in Fusarium. American J. Bot. 27: 64-67.
5. Stover, R. H. (1962). Fusarial wilt (Panama disease) of bananas and Musa species. Common Wealth Mycological Institute, Kew, Survey, UK, pp.17.

PLANT GROWTH PROMOTING OF *ZEA MAYS* L. IN METALLIFEROUS SOILS BY RHIZOSPHERIC BACTERIA

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Abstract:

Environmental rhizobacteria play an important role in bioremediation against various heavy metal pollutants in soil and wastewater. Phytoremediation is taken into account as a completely unique environmentally friendly technology that uses plants to get rid of or uptake heavy metals. The utilization of heavy metal tolerance plant growth-promoting rhizobacteria (PGPB) forms a key technology for increasing biomass preparation and defense of the plants to multiple heavy metals. The present study isolated 58 bacteria strains from soil and roots of maize plant rhizosphere irrigated with normal and wast water. The bacteria were tested for heavy metals resistance, salt tolerance, and PGP attributes. Pot culture experiments carried on under greenhouse environment and all data regarding growth attributes, physiological attributes, multiple heavy metal tolerant indexes, and accumulation of heavy metal in maize plant parts were recorded. Among the 58 isolated strains, 8 strains preliminary screened as multiple heavy metal resistance, salt tolerance, indole-3-acetic acid, phosphate solubilization, and siderophore production, and lastly, WW-40 strain was selected as the potent PGPR. Application of this strain in greenhouse condition significantly increased highest 52% of seed germination, 1078 % of vigour index, 68.57% of shoot length, 71% root length, 44.44% of shoot fresh weight, 50% of root fresh weight, 52.38% of shoot biomass, and 66.66% of root biomass excess as compared to heavy metal treatment maize seedling. The photosynthetic pigments of maize seedlings in heavy metal contaminated pots were reduced as compared to the consortium (WW-40 + Heavy metal) pots. The chlorophyll contents increased 68.75% excess in consortium with Zn than Zn inoculated pot. Similarly, the carotenoid contents increased 57.89% excess in Zn consortium pot and xanthophylls contents increased 65.62% excess in Ni consortium pot as compared to other metal treatment pots. The uptake of heavy metal by root and shoot of maize plants increased by inoculation of WW-40 strain as compared to plants in multiple heavy metal contamination without inoculation. Thus, the heavy metal tolerance bacterial isolate distinguish in our research have promise applications for rectifying metal contagion soils which is the potential of PGPR strain for both bioremediation and promotion of growth of crop plants have importance in the control of environmental contamination.

Keywords: Phytoremediation, Heavy metals, PGPR, metal accumulation, *Zeya mays*,

Introduction:

The growing urbanization and industrialization in the present world have resulted in serious environmental situations due to the discharge of pollutants especially heavy metals pollutants released regularly into the soil environment (Yahaghi *et al.*, 2018). The contamination

of normal water resources and agricultural soil is affected by multiple heavy metals due to the use of different fungicides, land chemical fertilizers, wastewater irrigation, and sewage sludge (Jadia and Fulekar, 2009; Akcil *et al.,* 2015). Thus, the protection of the environment from toxic effects is necessary for heavy metals through bioremediation (Glick, 2010). The traditional physicochemical approach for metal rectification such as, electrochemical processes, filtration, acid leaching, or ion exchange is costly and may not be very effective. The development of sustainable and environmentally friendly technologies is used to extract and remove toxic heavy metals from water and soil (Dixon,1996). The less cost-effective and environment-friendly method, bioremediation, clean-up the heavy metal through the use of microorganisms, plants, or other biological systems (Haferburg and Kothe, 2010). Among the bioremediation, phytoremediation is the most common method for soil remediation which utilizes plants to remove toxic metals from soils. The extraction of standard quantity of pollutants from superficial soil surfaces and water needs to hyper-accumulator plants but it grows slowly in metals stress because of toxicity of heavy metals and attains very low biomass (Zubair *et al.,* 2016; Atma *et al.,* 2017). So, the maintenance standard healthy state of heavy metal in soils can take years for reconstruction and depends on the concentration of heavy metals and types of soil of the particular area. At present, numerous plant species like an alpine weed (*Thlaspi caerulescens*), helianthus (*Helianthus annuus*), Indian mustard (*Brassica juncea*), and willow (*Salix* spp.), are being employed to soak up and accumulate serious metals from the soil (Solhi *et al.,* 2005). The capability of phytoremediation can be enhanced by the application of PGPR in metal contamination soils (Asghar *et al.,* 2013) which are termed as rhizoremediation apply plant-microbe for interactions to develop the potentiality of phytoremediation (Wu *et al.,* 2006). Root colonization capacity of numerous beneficial plant growth-promoting rhizobacteria promotes the growth of plants by the production of growth regulators like auxin, cytokinin, and gibberellin (El-Tarabily, 2008). Auxins enhance plant growth and metal uptake in metal stress conditions (Fassler *et al.,* 2010), and ethylene-induced seed germination or root growth by lowering the metal stress level. The most common PGPR strains *Bacillus* and *Pseudomonas* have been strongly utilized as metal uptake agents due to their high metal-binding capability (Chatterjee *et al.,* 2008). Morphological peculiarities of bacteria give them metal-binding capability because of the charge of the cell wall properties, the ratio of high surface-volume, protein of S-layer, and proteins of metal-binding (Velasquez *et al.,* 2009). PGPR bacteria multiple metal resistances in rhizosphere of plant considerably enhance metal adsorbing in plants and decrease metal toxicity (Jing *et al.,* 2007; Punamiya *et al.,* 2010). Plant growth attributes viz. growth hormones, siderophores, solubilizing phosphate by PGPR increase the availability of soluble iron, phosphate, IAA for improving total metal uptake by plants through reduction of metal stress in the rhizospheric soil (Kraemer *et al.,* 2006; Kuffner *et al.,* 2010). Metal bioavailability increased by metals solubilizes microbes and specific plants and which are not seen in a single PGPB (Jiang *et al.,* 2007; Rajkumar *et al.,* 2008).

The aims of this research are to i) screening of multiple heavy metals and salt tolerance rhizobacteria from contaminant maize field; ii) characterization, and pot experiment for determination of different plant growth parameters to increase phytoremediation capability in

metal toxicity soils; and iii) evaluation of enhancement of Photosynthetic pigment contents, metal index, and accumulation surrounds the maize root surface.

Materials and Methods:

1. Soil sampling and chemical analyses

The soil samples were collected from the rhizosphere of maize cultivated irrigated with industrial and municipal wastewater and irrigated with normal water maize fields in the English Bazar Block of Malda District. The collected sample was placed in a autoclaved container and brought to the laboratory for analysis. The physicochemical parameters of soil samples like pH, conductivity, total organic carbon, organic matter, total nitrogen content, total phosphorous content, total potassium content were analyzed using standard method (Rajan and Selvi Christy, 2010). The samples were dried and run through a 2 mm sieve before measuring pH, organic matter content, and the concentrations of available nitrogen (N), potassium (K), and phosphorus (P) as described (Ahmadpour *et al.*, 2015). Alkali N-proliferation method applied for soil available N detection whereas and available K and P measure with the ASI method. The K_2CrO_7 $2H_2SO_4$ oxidation method used to assess the soil organic matter. The air-dried soil samples were run through a 2 mm nylon sieve to extract heavy metals and dissolve by 1:2:2 (V:V:V) HNO_3:HCl:$HClO_4$. Vanadium (V), titanium (Ti), iron (Fe), zinc (Zn), nickel (Ni), lead (Pb), manganese (Mn), copper (Cu), chromium, arsenic (As), (Cr), and cadmium (Cd) were measured by inductively coupled.

2. Isolation and screening of multiple heavy metal tolerance rhizobacterial strains

To perform this experiment, 20 soil samples were collected from the maize rhizosphere of wastewater and normal water irrigated maize (*Zea mays* L., variety DHM –1) field, and samples were placed individually in plastic bags and brought to the laboratory. Soil plastic bags were open gently and 10 g of the soil sample were transferred into sterile flasks containing 90 ml of double sterile distilled water and kept on the rotary shaker at 150 rpm for 20 min. Then, serial dilution was made up from 10^{-1} to 10^{-9} using dilutions technique and 10 µl aliquot was inoculated in fresh sterile Nutrient Agar (NA) plate and incubated into 37°C biological incubator for 48 h. The strains were selected on the basis of their morphological features and tested for their resistance against five heavy metals viz. cadmium, lead, zinc, copper, and nickel. The heavy metal resistance assay was performed by spot-inoculating on nutrient agar medium amended with the respective metal salts. Minimum inhibitory concentration (MIC) values were calculated by adding $CdCl_2$, $Pb(NO_3)_2$, $CuSO_4$, $ZnCl_2$, and $NiCl_2$ in standard medium at 200, 400, 600, 800 and 1000 mg kg^{-1} heavy metal concentrations. After incubation at 28°C for 5 days, the MIC value of the viable colony-forming units (CFU) was observed after 48h of incubation at 30°C (Washington *et al.*, 1981).

3. Salt tolerance test of isolates

Assessment of salt tolerance test of all strains, NaCl (1–20%) amended with NA medium and allow to solidification in biological incubator. The overnight fresh culture of each isolate with a uniform population (5 x 10^8 cells ml^{-1}) was spotted on the fresh sterile NA agar plates and the petridishes were kept in the incubator at 37°C after sealing. Morphological characterization on the basis of colonies diameter and appearance of isolated strains were investigated after 3, 6, and 9 days incubation. Salt tolerance of each isolates to different percentages of salinity was

recorded by founding the quality of the bacterial colonies grown in the control plates (Noori *et al.,* 2018).

4. Plant growth promoting attributes of the isolates

Plant growth promoting parameters such as IAA production, solubilization of inorganic phosphate, and siderophore was assessed using different methods. IAA production measured by Sheng et al. (2008) methods of each PGPR isolated strain. The potent bacterial strains were cultured in 0.5 mg ml^{-1} L-tryptophan containing SMS medium for 4 days at 37°C at 200 rpm. Then, 2 ml Salkowski's chromogenic reagent was gently mixed with 1 ml cell suspension (Gordon and Weber, 1951) and place it dark for 30 min at 28°C and colour changes of the cell suspension was recorded. The ability of phosphate solubilization of isolated bacteria strains were determined by inoculating the bacterial culture in NBRIP medium at 28°C for 7 days and measurement the solubilization zone around each bacterial colony (Nautiyal, 1999). Production of Siderophore was determined using blue agar medium with chrome azurol S (CAS) and formation of halo zone around the colony was measures in terms of diameters (Schwyn and Neilands, 1987).

5. In vivo greenhouse experiment

5.1. Inoculum preparation and seed inoculation

The strains were cultured in a 250 ml conical flask individually containing 200 ml LB media and keep it at 37°C in the orbital shaking incubator (100 rpm) for 48 h. The fermenting broth was centrifuged at 6000 rpm for 6 min. The cell pellet was cleaned two times with sterile distilled water, and suspended in 10 ml sterile distilled water approximately 10^6 CFU/seed (O.D = 0.8), vortex, and used for seed treatment. Approximately 10–15 maize seeds were surface sterilized with 5% sodium hypochloride (NaoCl, Merk, India) for 1 min and washed thrice in sterile distilled water. Seeds were air-dried and soaked in bacterial suspension, and the preparation was stirred frequently for 5 min. Bacterized seeds were gently spread on a Petridish and air-dried overnight at room temperature. The total number of bacterial cells per seed was counted via serial dilutions and was set to approximately 10^6 CFU/seed (O.D = 0.8).

5.2. Experimental setup

The pots of earthen were filled with 8 kg soil with sandy clay loam texture having pH 7.64, organic matter 0.63%, EC 1.29 dS m1, saturation percentage 38.6%, extractable potassium 125.6 mg kg^{-1}, available phosphorous 7.5 mg kg^{-1} and while lead was not detectable in that soil. Before filling of the pot, this soil was polluted by lead using lead nitrate ($PbNO_3$) salt, Cadmium using cadmium chloride ($CdCl_2$) salt, Zinc using zinc chloride ($ZnCl_2$) salt, Cupper using copper sulphate ($CuSO_4$) salt and nickel using nickel chloride ($NiCl_2$) salt at a concentration of 200, 400, 600, 800 and 1000 mg kg^{-1}. Total 5 seeds were swan in each pot and kept at normal room temperature.

5.3. Analysis of plant growth attributes

Plant growth attributes such as seed germination and vigour index, shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight was recorded. Shoot and root dried in hot till the parts were lost their internal cell water.

The percentage of germination was evaluated with the following formula: Germination rate (%) = (number of seeds germinated/total number of seeds) × 100 Vigour index = % of germination × total plant length.

6. Assessment of heavy metal tolerance index

Evaluation of heavy metal tolerance index was measured according to the method of Balint *et al.,* 2002. The treated and control maize seedling samples were taken and dried at 50°C in a hot air oven.

The heavy metal tolerance index was calculated using the formula:

% Heavy metal tolerance index = Dry weight of Treated Plants / Dry weights of control Plants × 100

7. Determination of photosynthetic pigments

To an analysis of photosynthetic pigments, 100 mg leaf tissues collected from the heavy metal treated and control plant separately and then cut into small pieces and mixed with 7 ml of DMSO (dimethyl sulphoxide) in test tubes at 65°C for 3 h. After crushing the samples, DMSO was poured onto make up the volume up to 10 ml, and the absorbance of the filtered extract was recorded by UV-vis Spectrophotometer (UV-Vis 1800, Shimadzu, Japan) at 645 and 663 nm marking blank as pure DMSO (Sharma *et al.,* 2003). Extraction and estimation of Photosynthetic pigments from leaves of maize plants were ground with 80% acetone. Estimation of the chlorophylls and carotenoids has followed the method of Lichtenthaler, 1987. Photosynthetic pigments viz. total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids were expressed as mg/g of fresh leaf tissue. The absorbance for chlorophylls-a and -b and carotenoid was recorded at 663, 645, and 470 nm, respectively.

The content of xanthophylls estimation in maize leaves was done according to Lawrence (1990) method. Samples were oven-dried, crush and take 50 mg powdered to keep in a 100 ml flask. Then add the 30 ml of a combined extract of hexane (10 ml): acetone (7 ml) : absolute alcohol (6 ml) : toluene (7 ml)) was done and the flask was shaken for 15–20 min. After the addition of 40% methanolic KOH (2 ml) to the flask, it was then kept in the water bath (58°C) for 20–25 min and the samples were placed under dark conditions for 1 h. For this purpose, 30 ml of hexane and 10% sodium sulfate were mixed to a volume of 100 ml and then vigorous shaking for a minute. The flask was again incubated under dark conditions. After that, the upper portion was transferred into a 50 ml volumetric flask, and the volume was makeup using hexane, and absorbance was recorded at 474 nm.

8. Determination of multiple heavy metal accumulation

The seedlings of maize were collected and their separated roots and shoots parts were allowed to dry in the oven at 65°C for 48 hrs. The oven-dried samples were crushed to make powder and dissolve according to the Allen et al. (1976) method. For this purpose, 200 mg of dust form sample was taken and digested in aquaregia (H_2SO_4: HNO_3: $HClO_4$, v/v) in ratio 1:3:1 with help of beakers using hot induction plate. Then, dissolve samples were allowed to cooled and filtered through 0.22-µm pore containing nylon syringe filters. The dilution of samples was done by using double distilled water and the final volume make up to 50 ml. After that, these digested samples were stored at room temperature and estimated the roots and shoots heavy metal accumulation in plant through Atomic Absorption Spectrophotometer (Shimadzu 6200).

9. Statistical analysis

All the statistical analysis was performed using the statistical program SPSS v. 13.0 (SPSS, 2004).

Results:

1. Soil character analysis

Soil analysis result showed that different physicochemical properties of waste water irrigated soil significantly higher than normal water irrigated soil which was shown in Table-1. Similarly, the heavy metal content analysis of waste water irrigated soil showed higher ppm in context of normal water irrigated soil as shown in Table-1.

Table 1: Different physico-chemical properties of normal water and polluted water irrigated maize field soil

Physicochemical Properties		
Physicochemical properties	**waste water irrigatedsSoil**	**Normal water irrigated soil**
pH	6.6	5.1
Electrical Conductivity (m)	0.2	0.2
Organic Carbon (%)	2.64	0.84
Available N (kg/ha)	200.7	553.93
Available P(kg/ha)	116.16	3.24
Available K(kg/ha)	247.5	4.95
Heavy metals content (ppm)		
Heavy metals (ppm)	**Waste water irrigated soil**	**Normal water irrigated soil**
Lead (Pb)	14.99	3.36
Zinc (Zn)	11.47	5.08
Cadmium (Cd)	13.60	4.74
Cupper (Cu)	11.61	3.66
Nickel (Ni)	10.38	5.38
Calcium (ca)	10.75	2.0

2. Screening of heavy metal tolerance bacterial strains

Assessment of heavy metal tolerance of bacteria strains from maize rhizosphere as shown in Table- 2. A total of 48 bacteria isolated from maize rhizosphere irrigated with industrial and municipal wastewater showed were resistance to multiple heavy metals and 10 strains isolated from maize plant cultivated with normal water were mainly susceptible to heavy metals. The bacterial strains WW-09, WW-16, WW-22, WW-25, WW-40, WW-48, WW-51 and WW-55 were potent against multiple heavy metals.

Table 2: Heavy Metal tolerant assay of the isolated strains from normal water and polluted water irrigated maize field

Strains	Pb	Cd	Zn	Cu	NI	Co
NW- 30	–	–	–	–	–	–
NW- 31	–	–	–	–	–	–
NW- 32	–	–	–	–	–	–
NW- 33	–	–	–	–	–	–
NW-34	–	–	–	–	–	–
NW- 35	–	–	–	–	–	–
NW- 36	–	–	–	–	–	–
NW-37	–	–	–	–	–	–
NW-38	–	–	–	–	–	–
NW-39	–	–	–	–	–	–
WW- 40	+++	+++	++	++	++	++
WW- 41	+	+	+	+	+	+
WW- 42	+	+	+	+	+	+
WW- 43	+	+	+	+	+	+
WW- 44	+	+	+	+	+	+
WW- 45	+	+	+	+	+	+
WW- 46	+	+	+	+	+	+
WW- 47	+	+	+	+	+	+
WW- 48	++	+++	+	++	++	++
WW- 49	+	+	+	+	+	+
WW- 50	+	+	+	+	+	+
WW- 51	++	+++	++	+	++	++
WW-52	+	+	+	+	+	+
WW- 53	+	+	+	+	+	+
WW-54	+	+	+	+	+	+
WW-55	+++	+++	++	++	++	++
WW- 56	+	+	+	+	+	+
WW-57	+	+	+	+	+	+
WW- 58	+	+	+	+	+	+

Strains	Pb	Cd	Zn	Cu	NI	Co
WW- 1	+	++	+	++	+	+
WW- 2	+	+	+	+	+	+
WW- 3	+	++	+	+	+	+
WW- 4	+	+	+	+	+	+
WW- 5	+	++	++	+	+	+
WW- 6	+	++	+	+	+	+
WW- 7	+	+	+	+	+	+
WW- 8	+	+	+	+	+	+
WW - 9	+++	+++	++	++	++	++
WW- 10	+	+	+	+	+	+
WW- 11	+	+	+	+	+	+
WW- 12	+	+++	+	+	+	+
WW- 13	+	+	+	+	+	+
WW- 14	+	+	+	+	+	+
WW- 15	+	+	+	+	+	+
WW- 16	++	+++	+	++	+	++
WW- 17	+	+	+	+	+	+
WW- 18	+	+	+	+	+	+
WW- 19	+	+++	+	+	+	+
WW- 20	+	+	+	+	+	+
WW- 21	+	+	+	+	+	+
WW- 22	+++	+++	++	+++	++	++
WW- 23	+	+	+	+	+	+
WW- 24	+	+	+	+	+	+
WW- 25	++	+++	++	+	+	++
WW- 26	+	+	+	+	+	+
WW- 27	+	+	+	+	+	+
WW- 28	+	+	+	+	+	+
WW- 29	+	+++	+	+	+	+

Here +++ indicated highly heavy metal tolerant isolates, ++ indicated moderate heavy metal tolerant isolates and + indicated low heavy metal tolerant isolates. NW = Normal water irrigated soil and WW = waste water irrigated soil.

3. Salt tolerant test

The result of salt tolerance test of potent 8 bacterial strains shown in figure - 1. The result in this assessment showed that a significant percentage of rhizospheric isolates were resistant to high salinity percentages (up to 20 %). WW-40 isolated strains showed higher salt tolerance than

WW-09, WW-16, WW-22, WW-25, WW-40, WW-48, WW-51 and WW-55. In addition, due to high salts concentration, the rhizosphere isolates were not able to grow in 21% NaCl medium.

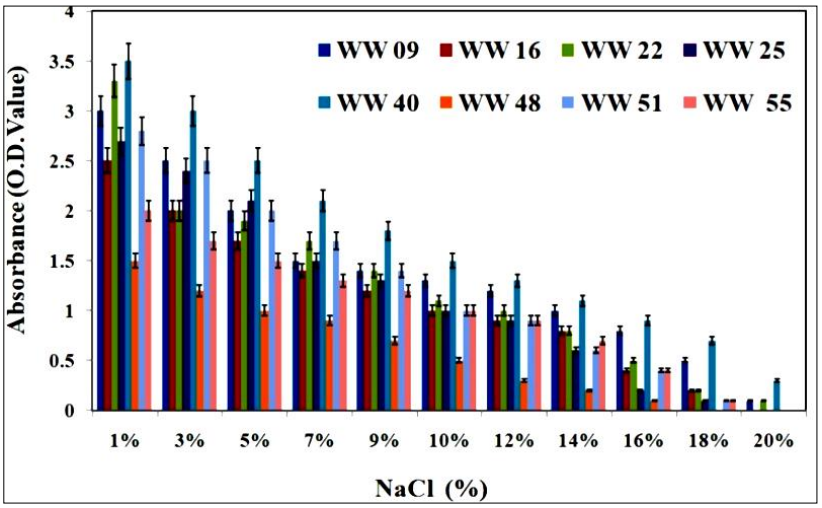

Figure 1: Salt tolerance (%) assay of maize (*Zea mays* L.) rhizospheric isolates irrigated with industrial and municipal wastewater

4. Maximum tolerable concentration assay of the potent PGPR Strains

The maximum tolerable concentration of multiple heavy metal tolerance 8 bacterial strains are shown in Table -3. According to the results, strains WW-09, WW-16, WW-22, WW-25, WW-40, WW-48, WW-51, and WW-55 showed good heavy metal tolerance potentiality against Pb, Cd, Zn, Cu, and Ni in MIC assay. All selected bacteria were observed to tolerate the tested heavy metals with different capabilities ranging from 1000 to 1600 mg L^{-1} for Pb, 600 to 1000 mg L^{-1} for Cd, 400 to 600 mg L^{-1} for Zn, 200 to 500 mg L^{-1} for Cu, and 300 to 1000 for Ni. Among the 8 strains, WW - 40 showed the highest multiple heavy metal tolerable capabilities than other isolated bacteria.

Table 3: Tolerance to heavy metals (MIC (mg L^{-1})

Strains	Pb	Cd	Zn	Cu	Ni
WW-09	1000	800	500	200	400
WW-16	1200	600	400	300	500
WW-22	1200	1000	600	300	500
WW-25	1400	1000	400	400	500
WW-40	1600	1200	600	500	1000
WW-48	1400	1000	500	400	500
WW-51	1200	800	400	300	400
WW-55	1000	600	400	200	300

5. Plant growth promoting activity

The results showed that the potent bacterial strains growing in medium amended with tryptophan were able to produce IAA as shown in Table - 4. Strain WW- 40 produced the highest (7.68 g/ml) whereas WW-16 produced the lowest (1.47 g/ml) IAA among the eight isolates tested. In addition to IAA production, all 8 strains exhibited the potential capability for phosphate solubilization (Table- 4). The highest phosphate solubilization was observed by the isolated strain WW- 40 (129.33 g/ml). The lowest quantity of phosphate solubilization was

observed by the isolate WW-22 (60.78 g/ml). Furthermore, the application of WW-40 PGPR strain in the presence of heavy metal for phosphate solubilization and IAA production was also investigated (Fig. 2). Results from this studies focuses that the existence of 3 mM zinc, 3 mM copper, and 3 mM Ni did not decrease the IAA production (Fig. 2a). Although, IAA production was decreased by Pb and Cd. Phosphate solubilization potensiality was inhibited by lead, cadmium, zinc, and copper (Fig. 2b) and not suppressed by Nickel.

Table 4: IAA production and phosphate solubilization by bacterial isolates without metal stress. All the values are mean of three replicates ± standard deviation (SD)

Strains	IAA	PS
WW-09	5.40 ± 0.23	72.98 ± 0.53
WW -16	1.47 ± 0.05	114.48 ± 1.86
WW - 22	5.43 ± 0.11	60.78 ± 0.20
WW -25	1.86 ± 0.04	102.42 ± 0.80
WW - 40	7.68 ± 0.45	129.33 ± 1.01
WW -48	2.52 ± 0.22	81.23 ± 0.92
WW - 51	1.85 ± 0.09	126.42 ± 0.62
WW -55	6.33 ± 0.25	106.58 ± 0.87

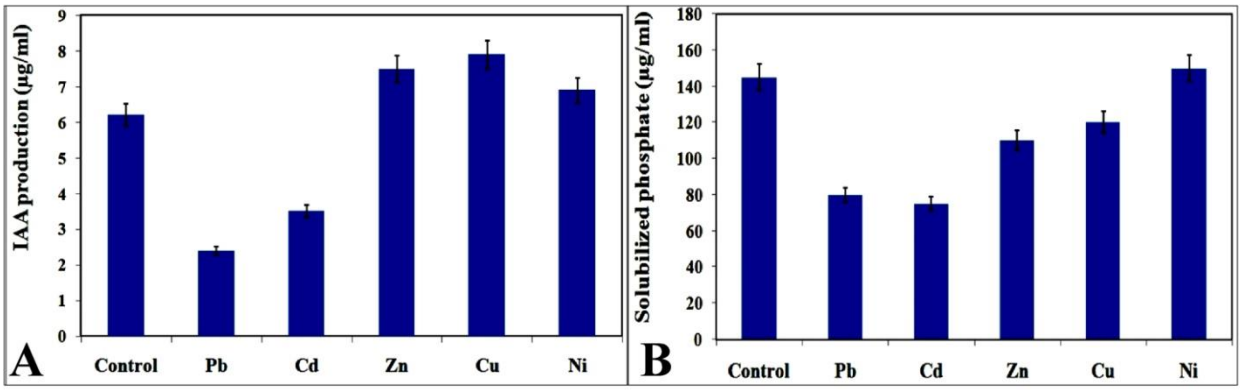

Figure 2: (a) IAA production by WW-40 and (b) phosphate solubilization by WW-40 in the presence of heavy metals. Control: absence of heavy metals.

The production of siderophores by the 8 multiple-metal tolerance bacterial strains was observed by the absorbance at 400 nm, as described in materials and methods. The production of siderophore was observed that WW 40 and WW 48 showing the maximum and minimum siderophore levels released in the supernatant (Fig. 3a). In addition, various in absorbances at 400 nm were shown between bacterial cultures grown in the presence versus the absence of iron. Furthermore, the effect of heavy metals on siderophore production by the bacterial isolates was showed in Figure. 3b. Results showed that except Nickel, all the heavy metals inhibited the production of siderophore in comparison to control.

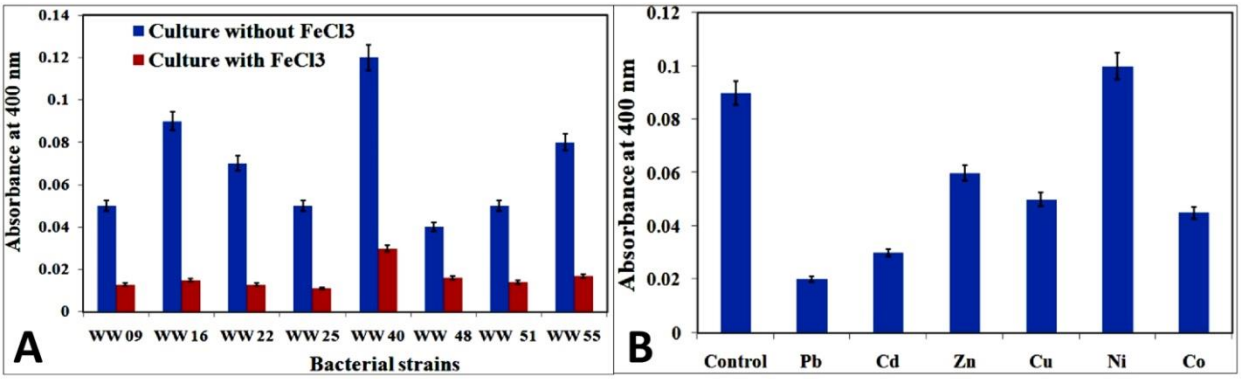

Figure 3: (a) Production of siderophore by the bacterial strains fermenting with and without $FeCl_3$, respectively. (b) Production of siderophore by WW-40 amended of heavy metals without $FeCl_3$. Control: absence of heavy metals

7. In vivo Plant growth parameters

The percentage of seed germination under various treatment conditions of Maize seedling shown in Figure- 4a which is focused on the potentiality of WW- 40 strain to induced maize seed germination in the presence of Pb, Cd, Cu, Zn, and Ni. Results showed that the highest percentage of seed germination occurs in only WW 40 strain in comparison to control. The application of WW- 40 strain with Pb, Cd, Cu, Zn, and Ni heavy metals, seed germination increases 2 fold approximately in comparison to Pb, Cd, Cu, Zn, and Ni inoculated pot. Similarly, vigour index of Maize seedling observed highest in case of WW 40 strain inoculated pot than control as shown in figure-4b. Application of consortium (WW 40 strain + heavy metals) showed that the vigour index increased approximately 5 fold of maize seedling compared with Pb, Cd, Cu, Zn, and Ni inoculated pots.

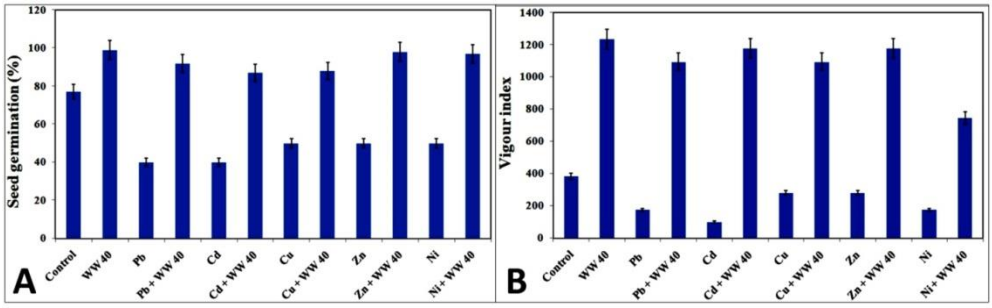

Figure 4: *In vivo* growth attributes of maize seedling
(a) Seed germination (%) (b) Vigour index

The effect of strain WW 40 up to 30 days maize seedlings under Pb, Cd, Cu, and Ni stress was assessed in terms of shoot length, root length, shoot fresh weight, root fresh weight, shoot biomass, and root biomass as shown in figure 4a-c. Results on root and shoot growth of maize seedling showed that the application of strain WW 40 increases with multiple heavy metal 2 fold length increased in Pb inoculated pot followed by 3 fold increased in Cd, 2.5 fold increased in Cu, 2.7 fold increased in Zn and 2.8 fold in Ni inoculated pots with comparison to Pb, Cd, Cu, Zn, and Ni inoculated pots as shown figure-5a. Similarly, shoot fresh weight increased excess 15 cm in Pb inoculated pot followed by 10 cm, 15 cm, 20 cm and 21 cm in Cd,

Cu, Zn and Ni inoculated pots. The root fresh weight observed highest excess growth 9 cm in Ni and Zn inoculates maize seedling whereas lowest growth 3cm by Cd and Cu inoculated pots as shown in Figure-5b.The shoot biomass excess increase by the application of strain WW 40 in Pb pot is 2.2 cm followed by 2 cm, 2.3 cm,3 cm and 2.7 cm in Cd, Cu, Zn and Ni pots whereas root biomass significantly increased 0.9 cm in Pb pot followed by 0.7 cm, 0.5 cm, 0.6 cm and 1.2 cm as shown in figure 5c.

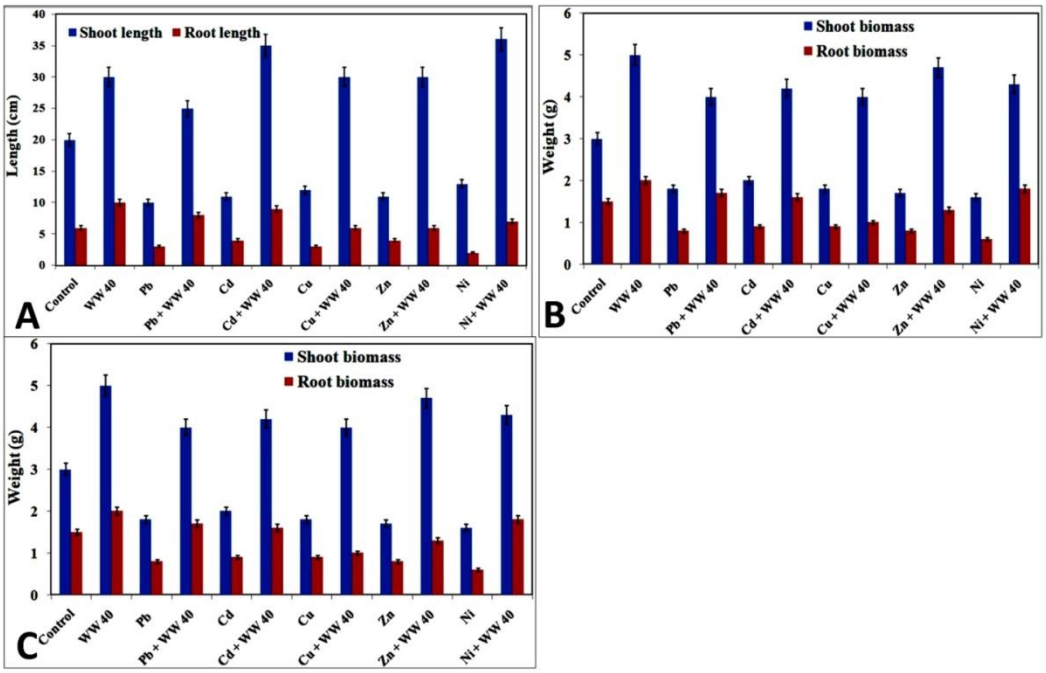

Figure-5: *In vivo* growth attributes of maize seedling. **(a) Root length and shoot length (b) Shoot fresh weight and root fresh weight (c) Shoot biomass and root biomass**

8. Photosynthetic pigment

The photosynthetic pigments were determined by analyzing total Chlorophyll, Carotenoid, and Xanthophyll contents as shown in Figure- 6a-c. Total chlorophyll content was observed lower 0.2 mg/g FW in Pb stress followed by 0.25 mg/g FW, 0.3 mg/g FW, 0.36 mg/g FW, and 0.4 mg/g FW in the presence of Cd, Cu, and Ni as compared to control seedlings as shown in Figure-6a. Application of strain WW 40 with Pb inoculated seedling observed that the chlorophyll content is significantly increased 1.5 -fold higher than Pb inoculated maize seedling. Similarly, in case of Cd, Zn, Cu and Ni inoculated pots, chlorophyll content increased 3- fold, 2.5 -fold, 1.5 -fold, and 2 -fold respectively for application of WW 40 strain. Total carotenoid content is observed lower 0.4 mg/g FW in Pb stress followed by 0.35 mg/g FW, 0.35 mg/g FW, 0.4 mg/g FW, and 35 mg/g FW in the presence of Cd, Cu, Zn, and Ni as compared to control seedlings as shown in figure-5b. Application of strain WW 40 in all heavy metal pot with maize seedling, carotenoid content is increased 1.75 -fold in Pb followed by 2.25- fold, 2.5 -fold, 2.25 -fold and 2- fold in Cd, Cu, Zn, and Ni inoculated pots. Total xanthophylls content was observed lower 10 mg/g FW in Pb stress followed by 9 mg/g FW, 12 mg/g FW, 11 mg/g FW, and 13 mg/g FW in the presence of Cd, Cu, Zn, and Ni as compared to control seedlings as shown in figure-5c. The content of xanthophylls is increased 2.2 -fold in Pb- inoculated pot, 3 -fold in Cd, 2- fold

in Cu, 2.3 -fold in Zn by the application of PGPR strain WW 40 in all heavy metal inoculated pots.

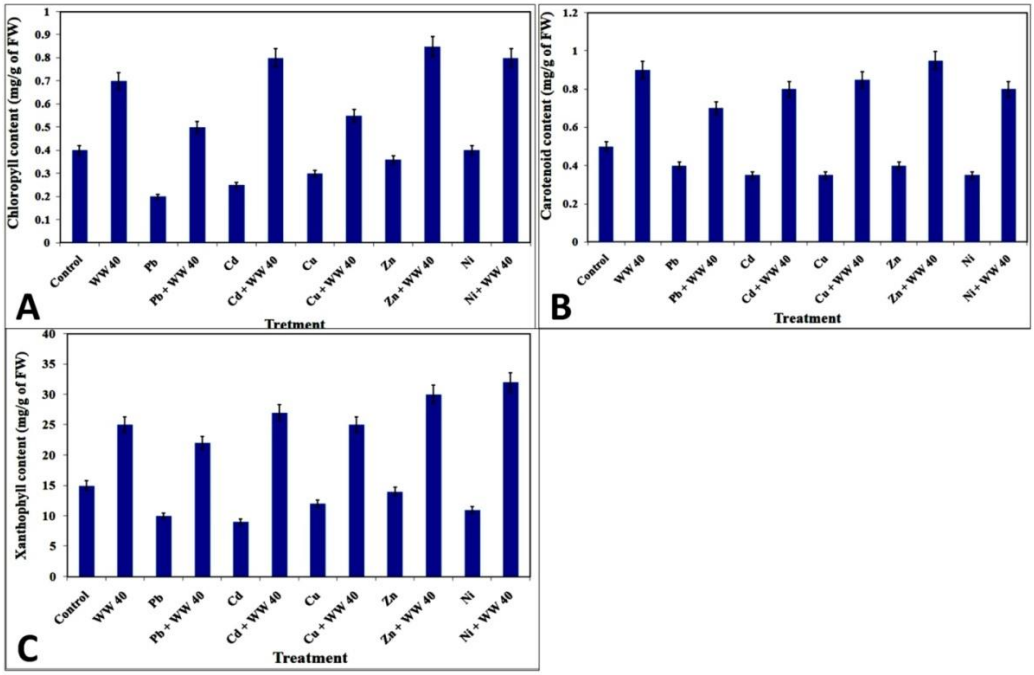

Figure 6: Effect of PGPR strains WW 40 on photosynthetic pigments (A) Chlorophyll content (B) Carotenoid content (C) Xanthophyll content in 20-days old *Zea maize* seedlings under five heavy metal stresses.

9. Heavy metal tolerance Index and accumulation.

The tolerance index of maize plant against Pb, Cd, Cu, Zn, and Ni heavy metal was observed 100% in control seedling and 57.77 % in Pb stress followed by 64.44 %, 60 %, 55.55 and 48.88 % in the presence of Cd, Cu, Zn, and Ni as compared to control seedlings as shown in figure-7. The tolerance index of WW 40 strain amended with Pb, Cd, Cu, Zn, and Ni stressed seedling are 137.77%, 142.22%, 111.11%, 133.33% and 135.55% respectively.

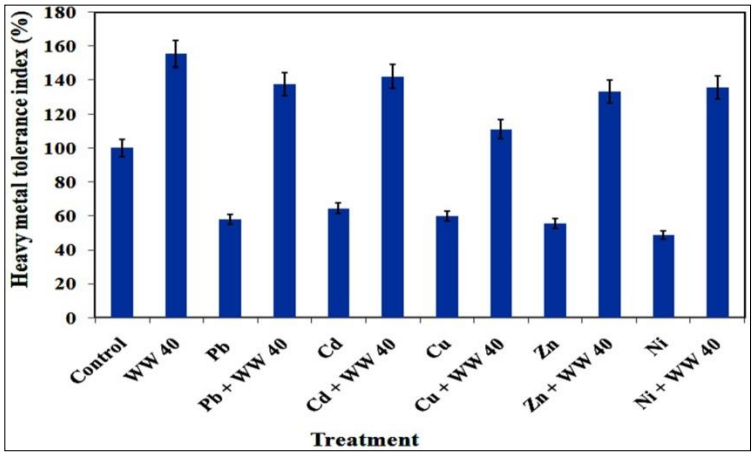

Figure 7: Heavy metal tolerance index in 20 -days old *Zea mays* seedlings under five heavy metal stresses

Heavy metal accumulation in the shoot of maize plant observed to the highest reduction in Cd inoculated pot and lowest reduction in Cu inoculated pot by the application of PGPR strain

WW 40 as compared than only heavy metal inoculated pot as shown in figure-8a. Similarly, 50% Cd accumulation reduces by the PGPR strain WW 40 which is highest and lowest reduction observed in case of Pb as shown in figure - 8b.

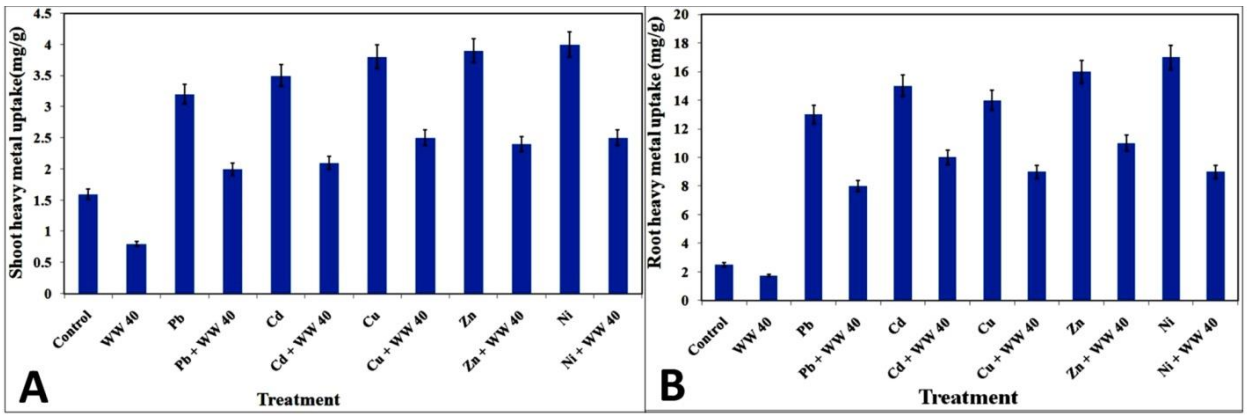

Figure 8: Effect of WW 40 on (A) Shoot metal uptake (B) Root metal uptake

Discussion:

In recent years, PGPR is widely utilized for phytoremediation of metaliferous soils of the agricultural field which help the growth promotion of crops under stress conditions and reduce the heavy metals toxicity of crop plants in different ecosystems (Chiboub *et al.,* 2018; Abou-Shanab *et al.,* 2020). Production of crop losses increase the Pb, Cd, Zn, Cu, and Ni content in the soil that affects on the growth of the crop plants in the agricultural field (Abdelkrim, *et al.,* 2020). According to Kotoky *et al.,* 2019 reports, it has been well established that no plant can grow well without the presence of microorganisms. The present study focuses on the rhizospheric bacteria colonization on maize plant root surface to help the growth and tolerance of these plants from environmental stresses. There is no toxicity effect of heavy metals on maize plants observed and so plants become healthy. The previous report revealed that PGPR adapted to heavy metal toxicity for their survival in places contaminated metaliferous soils (Balseiro-Romero *et al.,* 2017). The potency of heavy metal resistance of contaminated environments isolates has higher resistance than normal areas (Dabrowska *et al.,* 2017). Hence, it can be expected that wastewater irrigated maize plants rhizospheric bacterial isolates are inhabitant in highly metal–resistant soil, and their population is affected by biological and non–biological agents such as heavy metal stress (Yang *et al.,* 2020). The maize field isolated strains of this study also showed remarkable tolerance to salinity. Previous reports showed that few halophilic bacteria resistant to heavy metals (Voice *et al.,* 2016) and six heavy metal resistant bacteria isolates viz. *Chryseobacterium indoltheticum, Pseudomonas helmanticensis, Cupriavidus oxalaticus, Bacillus almalaya, Bacillus mycoides,* and *Acinetobacter tjernbergiae* were able to tolerate 1–7% salinity (Jiang *et al.,* 2017). Salt tolerance reports observed that these isolated strains have the potential to be used in area of highly salinity and metalliferous polluted areas (Figure 1).

In this study 8 rhizospheric bacterial isolates showed different PGP attributes such as IAA production, siderophore and phosphate solubilization (Table - 4; figure- 2 and 3). Past studies on rhizospheric bacteria from various crop plants were potentially tolerate to heavy

metals also showed these PGP traits (Etesami *et al.,* 2018). Multiple heavy metals–stressed plants associated with bacterial isolates actively shown production such PGP properties of *Zea mays* cultivated with wastewater in this study. IAA production by the 8 strains is different quantities. The previous report documented that 80% of rhizospheric bacteria isolated from various plants rhizosphere had the capability to synthesis IAA as secondary metabolites (Ma *et al.,* 2016b). However, IAA production can help the bacteria to interact with plant that has no specific role in bacterial cells (Etesami *et al.,* 2015). Rhizospheric bacteria attached with the epidermal cell of the root surface and increase the number of root hairs initiation by the production of bacterial IAA which loosens the cell wall of the plant and increases the number of roots that help the take nutrients from the surrounding soil (Linu *et al.,* 2009). Siderophore production is one of the most essential characters of rhizobacteria which is synthesized under very low iron stress, act as specific ferric iron-chelating agents and make them more powerful in challenge with other microorganisms in the environment and helping plant-bacteria for root colonization (Ma *et al.,* 2016a). In this study PGPR significantly produce siderophore in the heavy metal condition which is shown in Figure-3. The most potent strain WW40 produces the highest siderophore than control under multiple heavy metal conditions. Phosphorus is a necessary mineral nutrient that helps various physiological roles for plant growth and development. However, a large amount of total phosphate stock in soils but plants can be utilized a poor amount of the total phosphorus terms as plant-available phosphorus (Stevenson and Cole, 1999). The soil stress due to heavy metal can also a barrier to the absorption of plant-available phosphate and suppressed plant growth. Although, Phosphate availability could also be in the soil enhance the solubilization potentiality of phosphate by many PGPR through increasing the mobile of inorganic P to available phosphate (Wood *et al.,* 2016; Rafique *et al.,* 2017). The amounts of absorption of soil phosphorous by plants enhance and improved plant growth promotion. We found that WW-34, WW-36, WW-37, and WW-58 significantly increased the total P content in shoots and roots of *Zea mays* growing in WW-34, WW-36, WW-37, and WW-58 contaminated soil compared to control plants (Figure-2b). Seed bacterization of *Zey mays* with WW-34, WW-36, WW-37, and WW-58 strains significantly increased the rate of the seed germination and vigour index in comparison to the untreated control (Figure- 4a-b). The percentage (92%) of seed germination observed in Pb inoculated pot with WW-40 strain is 52% greater than Pb inoculated pot only (40%). The application of WW-40 strain in Cd, Zn, Cu, and Ni pots significantly increased 47%, 38%, 38, 47%. Similarly, the vigour index of *Zea mays* seedling significantly increase 918%, 1078%, 812%, 896%, and 569% by the application of WW-40 strain in Pb, Cd, Cu, Zn, and Ni inoculated pots. Previous research focuses on the inhibition of seed germination in various cereal crops such as rice, wheat, and barley in response to different heavy metals which is probably due to the morphological and physiological changes in roots that result in reducing heavy metal tolerance (Mahmood *et al.,* 2007**)**. Our results also demonstrated that there was a significant increase in plant growth parameters like shoot and root length, shoot and root fresh weight, and biomass (Figure. 5a–c). The shoot length of maize seedling increased 60%, 68.57%, 66.66%, 63.33% and 91.66% and root length increased 62.5%, 55.55%, 50%, 33.33% and 71.42% excess by the application of the strain WW 40 in Pb, Cd, Cu, Zn, and Ni inoculated pots. The highest shoot fresh weight increased excess 46.51%, root fresh

weight 50%, and root biomass (63.93%) observed in Ni inoculated pots and shoot biomass 63.82% in Zn inoculated pot by application of WW 40 strain. The effect of heavy metals on seeds with various growth abnormalities viz. germination, reduced root, and shoot elongation (; Ahmad and Ashraf 2011; Pourrut *et al.,* 2011) reported by earlier researchers. The pigments content of *Zea mays* seedling (Chlorophyll 'a', 'b', Xanthophyll and Carotenoids content) are reduced by Pb, Cd, Zn, Cu, and Ni contamination pots (Figure-6). The pigments content might be reduced due to the insertion of multiple heavy metals wiyhin the phytoporphyrin ring of the pigments chlorophyll and decrease the production of chlorophyll (Nyitrai *et al.,* 2002; Jaleel *et al.,* 2009). The synthesis of chlorophyll molecule decrease either by reducing the potentiality of chlorophyllase enzyme or lowering the adsorption of Fe and Mg by plants (Sharma and Dube, 2005). Cd, Zn, Cu and Ni also degraded the chlorophyll molecule (Dogan *et al.,* 2009). The photosynthetic pigments of crop plants were observed to enhancement by the application of plant growth-promoting bacteria that increases nutrient uptake in plants through phosphate solubilization and exudating essential substances that play a crucial role in synthesis of photosynthetic pigments necessity for light-harvesting complex and its photo assimilation (Tanaka *et al.,* 1998). The chlorophyll levels increasing due to inoculation of *Klebsiella pneumoniae* in *V. mungo* under Cd stress (Dutta *et al.,* 2018). The chlorophyll contents of *Zea mays* plants also increased by the application of *Azotobacter chrococcum* with Cu and Pb (Rizvi and Khan, 2018). Heavy metal tolerance index showed that consortium (Pb + WW 40) applied *Zea mays* seedling tolerate 43.83% higher than Pb inoculated pot. Similarly, the application of in Cd, Cu, Zn, and Ni pot significantly increases the metal tolerance index 29.64%, 56%, 47.82% respectively (Figure 7). The result also signifies that treatment of maize seedlings with microbial strains WW 40 in each heavy metal pot mitigates Pb, Cd, Cu, Zn, and Ni and reduce the heavy metal tolerance index. The application of *Methylobacterium oryzae* and *Burkholderia* sp. with Cd and Ni inoculated tomato seedlings decrease the Heavy metal tolerance index uptake (Madhaiyan *et al.,* 2007). In the current study, PGPR microorganisms are reduced heavy metal accumulation in shoot of maize plant observed at 37.5% in Pb inoculated pot, 40% in Cd, 34.21% in Cu, 38.46% in Zn and 37.8% in Ni pot as compared to Pb, Cd, Cu, Zn, and Ni pot as shown in Figure-8a. Similarly, in case of root, heavy metal accumulation lowered 38.4% in Pb inoculated pot, 40% in Cd, 50% in Cu, 43.75% in Zn, and 47.05% in Ni pot by the application of PGPR strain WW 40 as shown in Figure-8b. The mechanism of accumulation of Pb, Cd, Cu, Zn, and Ni reduction could be enhancement by the potential application of rhizobacteria. Previous studies suggested that *Z. mays* when contaminated with *A. chroococcum* bacterium lowerd Cu and Pb accumulation in plant parts which is most likely due to the synthesis of various metabolites, protons and exudates that act as metal chelators and immobilise Pb (Rizvi and Khan; 2018). Moreover, it was suggested that *Bacillus megaterium,* a metal tolerant strain, decrease the Ni translocation (Rajkumar *et al.,* 2013), and As-resistance Exiguobacterium decrease As translocation in *Vigna radiata* plants by accumulation at the root parts (Pandey and Bhatt, 2016). It has been observed that *Acinetobacter lwofi* promotes the growth and reduces uptake in *V. radiata* (Das and Sarkar, 2018).

Conclusions:

The maize field isolated multi-metals resistant and salt tolerance bacterium appeared to be potent plant growth-promoting that would turn out IAA, siderophores, solubilize the phosphate. The present study complete that the rhizobacteria WW 40 strain enhance the rate of seed germination and vigour index in the metalliferous soil and promoted growth of maize seedling in terms of root length, shoot length, root fresh weight and shoot fresh, root biomass and shoot biomass subjected to Pb, Cd, Cu, Zn, and Ni metallic element stress. Application of the strain in Pb, Cd, Cu, Zn, and Ni pots observed a significant increase in the photosynthetic pigment content of seedlings like chlorophyll, carotenoid, and xanthophylls. The Pb, Cd, Cu, Zn, and Ni metal accumulation capability reduced by the seedling found in the presence of WW 40 strain in the rhizosphere. The decreased levels of Pb, Cd, Cu, Zn, and Ni resulted in the alleviation of Pb, Cd, Cu, Zn, and Ni toxicity by decreasing its bioavailability. Therefore, all these traits act as a driving force in enhancing the growth of plants in a metal-polluted environment. The present study, therefore, projects the contribution of micro-organisms in decreasing Pb, Cd, Cu, Zn, and Ni toxicity and accumulation, implicating their roles for achieving the goal of lower Pb, Cd, Cu, Zn, and Ni concentrations in Maize plants with better growing conditions. It could be also a good choice for application in microbially assisted phytoremediation approaches for depollution of multi-metals contaminated soils.

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References:

1. Yahaghi, Z., Shirvani, M., Nourbakhsh, F., Pena, T. C.D., Pueyo, J.J., Talebi, M., Isolation and Characterization of Pb-Solubilizing Bacteria and Their Effects on Pb Uptake by *Brassica juncea*: Implications for Microbe Assisted Phytoremediation, J. Microbiol. Biotechnol, 2018, Vol. 28, No.7, pp.1156–1167.
2. Jadia, C.D., and Fulekar, M.H., Phytoremediation of heavy metals: recent techniques, Afr.J. Biotechnol , 2009, Vol. 8, pp. 921–8.
3. Akcil, A., Erust, C., Ozdemiroglu, S., Fonti, V., Beolchini, F., A review of approaches and techniques used in aquatic contaminated sediments: metal removal and stabilization by chemical and biotechnological processes. J. Clean. Prod, 2015, Vol. 86, pp. 24–6.
4. Glick, B.R., Using soil bacteria to facilitate phytoremediation, Biotechnol. Adv, 2010, Vol. 28 pp. 367–74.
5. Dixon, B., Bioremediation is here to stay, ASM News, 1996, Vol. 62, pp. 527–528.
6. Haferburg, G., Kothe, E., Metallomics: lessons for metalliferous soil remediation, Appl. Microbiol. Biotechnol., 2010, Vol. 87, pp. 1271–1280.
7. Atma, W., Larouci, M., Meddah, B., Benabdeli, K., Sonnet, P., Evaluation of the phytoremediation potential of Arundo donax L. for nickel-contaminated soil. Int. J. Phytorem, 2017, Vol. 19 No.4, pp. 377-386.

8. Zubair, M., Shakir, M., Ali, Q., Rani, N., Fatima, N., Farooq, S., Nasir, I.A.,. Rhizobacteria and phytoremediation of heavy metals. Environ. Technol. Rev. 2016, Vol.5 No.1, pp.112-119.
9. Asghar, H.N., Ishaq, M., Zahir, Z.A., Khalid, M., Arshad, M., Response of radish to integrated use of nitrogen fertilizer and recycled organic waste. Pak. J. Bot. 2006, Vol. 38 No. 3, pp. 691-700.
10. El-Tarabily, K.A., Promotion of tomato (*Lycopersicon esculentum* Mill.) plant growth by rhizosphere competent 1-aminocyclopropane-1-carboxylic acid deaminase-producing streptomycete actinomycetes. Plant Soil, 2008, Vol. 308, pp.161-174.
11. Fassler, E., Evangelou, M.W., Robinson, B.H., Schulin, R., Effects of indole-3- acetic acid (IAA) on sunflower growth and heavy metal uptake in combination with ethylene diamine disuccinic acid (EDDS). Chemosphere, 2010, Vol. 80, pp. 901- 907.
12. Solhi, M., Shareatmadari, H., Hajabbasi, M., Lead and zinc extraction potential of two common crop plants, *Helianthus annuus* and *Brassica napus*, Water Air Soil Pollut, 2005, Vol.167 pp. 59–71.
13. Wu, C.H., Wood, T.K., Mulchandani, A., Chen, W., Engineering plant-microbe symbiosis for rhizoremediation of heavy metals, Appl. Environ. Microbiol, 2006, Vol.72 pp.1129–1134.
14. Chatterjee, S.K., Bhattacharjee, I., Chandra, G., Biosorption of heavy metals from industrial waste water by *Geobacillus thermodenitrificans*, J. Hazard. Mater, 2010, Vol. 175 pp.117–125.
15. Velasquez, L., Dussan, J., Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*, J. Hazard. Mater, 2009, Vol. 167 , pp. 713–716.
16. Jing, Y.D., He, Z.L., Yang, X.E., Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils, J. Zhejiang Univ. Sci. B, 2007, Vol. 8 pp. 192–207.
17. Punamiya, P., Datta, R., Sarkar, D., Barber, S., Patel, M., Das, P., Symbiotic role of *Glomus mosseae* in phytoextraction of lead in vetiver grass [*Chrysopogon zizanioides* (L.)], J. Hazard. Mater. 2010, 177pp. 465–474.
18. Kuffner, M., De Maria, S., Puschenreiter, M., Fallmann, K., Wieshammer, G., Gorfer, M., Strauss, J., Rivelli, A.R., Sessitsch, A., Culturable bacteria from Zn and Cd accumulating *Salix caprea* with differential effects on plant growth and heavy metal availability, J. Appl. Microbiol, 2010, Vol.108, pp. 1471–1484.
19. Kraemer, S.M., Crowley, D.E., Kretzschmar, R., Siderophores in plant iron acquisition: geochemical aspects, Adv. Agron, 2006, Vol. 91, pp. 1–46.
20. Jiang, C.Y., Sheng, X.F., Qian, M., Wang, Q.Y., Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil, Chemosphere, 2008, Vol. 72, pp.157–164.
21. Rajkumar, M., Ma, Y., Freitas, H., Characterization of metal-resistant plant growth promoting *Bacillus weihenstephanensis* isolated from serpentine soil in Portugal, J. Basic Microbiol, 2008, Vol. 48, pp. 500–508.

22. Rajan, S., and Selvi Christy. R., Experimental procedures in life sciences. Anjana book press, Chennai. 2010, pp. 265-275.
23. Ahmadpour, P., Ahmadpour, F., Sadeghi, S., Tayefeh, F. H., Soleimani, M., Abdu, A. B., Evaluation of Four Plant Species for Phytoremediation of Copper-Contaminated Soil. Soil Remediation and Plants, 2015, pp. 147–205.
24. Washington, J.A., Sutter, V.L., Dilution tests procedures. In: Balows A, Hausler WJ, Truant JP, Lennette EH, editors. Manual of clinical microbiology, Washington D.C, American Society for Microbiology, 1981, pp. 549–55.
25. Noori, F., Etesami, H., Najafi Zarini, H., Khoshkholgh-Sima, N.A., Hosseini Salekdeh, G., Alishahi F., Mining alfalfa (M*edicago sativa* L.) nodules for salinity tolerant non-rhizobial bacteria to improve growth of alfalfa under salinity stress, Ecotoxicol. Environ. Saf., 2018, Vol. 162 pp. 129-138.
26. Sheng, X. F., Xia, J. J., Jiang, C. Y., He, L. Y. and Qian, M. Characterization of Heavy Metal-resistant Endophytic Bacteria from Rape (*Brassica napus*) Roots and Their Potential in Promoting the Growth and Lead Accumulation of Rape. Environ. Pollut., 2008, Vol. 156 No.3, pp. 1164-1170.
27. Gordon, S.A., Weber, R.P.,. Colorimetric estimation of indoleacetic acid. Plant Physiol, 1951, Vol. 26, pp. 192–195.
28. Belimov, A. A., Hontzeas, N., Safronova, V. I., Demchinskaya, S. V., Piluzza, G., Bullitta, S., Glick, B. R., Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biology and Biochemistry, 2005, Vol.37, No. 2, pp. 241–250.
29. Nautiyal, C.S. and Mebta, S.B. An Efficient Method for qualititative screening of phosphate – Solubilizing Bacteria. Microbiology, 2001,Vol. 43, pp. 51-56.
30. Schwyn, B., Neilands, J.B., Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry ,1987,Vol. 160, pp. 47–56.
31. Balint, A. F., Kovács, G., Sutka, J., Copper tolerance of *Aegilops*, *Triticum*, *Secale* and *triticale* seedlings and copper and iron contents in their shoots. Acta Biol. Szeged, 2002, Vol. 46, pp. 77–78 .
32. Lichtenthaler, H.K., Chlorophylls and carotenoids: Pigments of photosynthetic membranes, Method Enzymol., 1987, No. 148, pp. 350–382.
33. Lawrence., J. Determination of total xanthophyll and marigold oleoresin. J. AOAC Int. 1990, Vol. 2, pp. 970–975.
34. Allen, S. E., Grimshaw, H. M. Rowland, A. P., Chemical analysis. In: Chapman, S. B. (Ed.), Methods in Plant Ecology. Blackwell Scientifc Publications, Oxford – London, 1976, pp.335–335.
35. Chiboub, M., Jebara, S.H., Saadani, O., Fatnassi, I.C., Abdelkerim, S., Jebara, M., Physiological responses and antioxidant enzyme changes in *Sulla coronaria* inoculated by cadmium resistant bacteria. J. Plant Res. 2018, Vol. 131, pp. 99–110.
36. Abou-Shanab, R.A.I., Mathai, P.P., Santelli, C., Sadowsky, M.J., Indigenous soil bacteria and the hyperaccumulator Pteris vittata mediate phytoremediation of soil contaminated with arsenic species. Ecotoxicol. Environ. Saf. 2020, Vol. 195, 110458.

37. Abdelkrim, S., Jebara, S.H., Saadani, O., *et al.,* In situ effects of Lathyrus sativusPGPR to remediate and restore quality and fertility of Pb and Cd polluted soils. Ecotoxicol. Environ. Saf. 2020, Vol. 192, 110260.
38. Kotoky, R., Nath, S., Kumar Maheshwari, D. *et al.* Cadmium resistant plant growth promoting rhizobacteria *Serratia marcescens* S2I7 associated with the growth promotion of rice plant. *Environmental Sustainability* **2,** 135–144 (2019).
39. Balseiro-Romero, M., Gkorezis, P., Kidd, P.S., *et al.,* Use of plant growth promoting bacterial strains to improve *Cytisus striatus* and *Lupinus luteus* development for potential application in phytoremediation. Sci. Total Environ. 2017, Vol. 581, pp. 676–688.
40. Dabrowska, G., Hrynkiewicz, K., Trejgell, A., Baum, C., The effect of plant growth promoting rhizobacteria on the phytoextraction of Cd and Zn by *Brassica napus* L. Int. J. Phytoremediation 2017,Vol. 19, 597–604.
41. Yang, X., Liu, L.H., Tan, W.F., Liu, C.S., Dang, Z., Qiu, G.H., Remediation of heavy metal contaminated soils by organic acid extraction and electrochemical adsorption. Environ. Pollut. 2020, Vol. 264, 114745.
42. Voica D.M., Bartha L., Banciu H.L., Oren A. Heavy metal resistance in halophilic Bacteria and Archaea. FEMS Microbiol. Lett. 2016, Vol. 363.
43. Jiang J., Pan C., Xiao A., Yang X., Zhang G. Isolation, identification, and environmental adaptability of heavy-metal-resistant bacteria from ramie rhizosphere soil around mine refinery. 3 Biotech, 2017, Vol. 7, pp. 5.
44. Etesami H. Bacterial mediated alleviation of heavy metal stress and decreased accumulation of metals in plant tissues: mechanisms and future prospects. Ecotoxicol. Environ. Saf. 2018, Vol.147,pp.175–191.
45. Ma, Y., Rajkumar, M., Zhang, C., Freitas, H., Inoculation of Brassica oxyrrhina with plant growth promoting bacteria for the improvement of heavy metal phytoremediation under drought conditions. J. Hazard Mater, 2016b, Vol. 320, pp.36–44.
46. Etesami H., Alikhani H.A., Hosseini H.M. Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. MethodsX. 2015, Vol. 2, pp.72–78.
47. Linu, M. S., Stephen, J., Jisha, M. S., Phosphate solubilizing *Gluconacetobacter* sp., *Burkholderia* sp. and their potential interaction with cowpea (*Vigna unguiculata* L. Walp.). Int. J. Agric. Res. 2009, Vol.4, No.2, pp.79–87.
48. Ma, Y., Oliveira, R.S., Freitas, H., Zhang, C., Biochemical and molecular mechanisms of plant-microbe-metal interactions: relevance for phytoremediation. Front. Plant Sci. 2016a,Vol. 7, pp.1–19.
49. Stevenson, F.J., Cole, M.A., Cycles of soil: carbon, nitrogen, phosphorus, sulfur. In: Micronutrients, second ed. Wiley, New York, USA, 1999.
50. Wood, J.L., Tang, C.X., Franks, A.E., Microbial associated plant growth and heavy metal accumulation to improve phytoextraction of contaminated soils. Soil Biol. Biochem. 2016,Vol. 103, pp. 131–137.

51. Rafique, M., Haque, K., Hussain, T., Amna, C., Javed, H., Biochemical Talk in the Rhizoshperic Microbial Community for Phytoremediation. 2017, Nova Science Publishers. NY, ISBN 978-1-53611-047-0.
52. Mahmood, T., Islam, K. R. and Muhammad, S. Toxic effects of heavy metals on early growth and tolerance of cereal crops. Pakistan J. Bot. 2007, Vol. 39, No.2, pp.451.
53. Ahmad, M.S., Ashraf, M., Essential roles and hazardous effects of nickel in plants. Rev Environ Contam Toxicol, 2011, Vol. 214, pp. 125–67.
54. Pourrut, B., Shahid, M., Dumat, C., Winterton, P., Pinelli, E., Lead uptake, toxicity, and detoxification in plants. Rev Environ Contam Toxicol. 2011, Vol. 213, pp.113–36.
55. Nyitrai, M., A.G. Szent-Gyorgyi and M.A. Geeves, A kinetic model of the co-operative binding of calcium and ADP to scallop (*Argopecten irradians*) heavy meromyosin. Biochem. J., 2002,Vol. 365,pp. 19-30.
56. Jaleel, C.A., K. Jayakumar, Z. Chang-Xing and M. Iqbal, Low concentration of cobalt increases growth, biochemical constituents, mineral status and yield in *Zea Mays*. J. Sci. Res., 2009. Vol. 1, pp.128-137.
57.Sharma, P. and R.S. Dubey, Lead toxicity in plants. Braz. J. Plant Physiol., 2005,Vol. 17,pp.35-52.
58. Dogan, M., Saygideger, S. D., Colak, U. Effect of lead toxicity on aquatic macrophyte elodea canadensis michx. Bulletin of Environmental Contamination and Toxicology, 2009,Vol. **83, No.** 2, pp. 249– 254.
59. Tanaka, A., Ito, H., Tanaka, R., Tanaka, N. K., Yoshida, K., Okada, K. Chlorophyll a oxygenase (CAO) is involved in chlorophyll b formation from chlorophyll a. Proceedings of the National Academy of Sciences, 1998, Vol. 95, No.21, pp.12719–12723.
60. Dutta, P., Karmakar, A., Majumdar, S., Roy, S. *Klebsiella pneumoniae* (HR1) assisted alleviation of Cd (II) toxicity in *Vigna mungo*: a case study of biosorption of heavy metal by an endophytic bacterium coupled with plant growth promotion. Eur. Med. J. Environ. Integ. 2018, Vol. 3, No.1, pp. 27.
61. Rizvi, A., Khan, M. S., Heavy metal induced oxidative damage and root morphology alterations of maize (*Zea mays* L.) plants and stress mitigation by metal tolerant nitrogen fxing *Azotobacter chroococcum*. Ecotoxicol. Environ. Saf. 2018, Vol. 157, pp.9–20.
62. Madhaiyan, M., Poonguzhali, S., Sa, T., Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). Chemosphere 2007, Vol.69, No. 2, pp. 220–228.
63. Rajkumar, M., Ma, Y., Freitas, H., Improvement of Ni phytostabilization by inoculation of Ni resistant *Bacillus megaterium* SR28C. J. Environ. Manag, 2013, Vol.128, PP. 973–980.
64. Pandey, N., Bhatt, R., Role of soil associated Exiguobacterium in reducing arsenic toxicity and promoting plant growth in *Vigna radiata*. Eur. J. Soil Biol. 2016, Vol. 75, pp. 142–150.

A CONTEMPORARY STUDY ON SOME IMPORTANT STAINING TECHNIQUES

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Abstract:

The history of histology indicates that there have been significant changes in the techniques used for histological staining through chemical, molecular biology assays and immunological techniques, collectively referred to as histochemistry. Early histologists used the readily available chemicals to prepare tissues for microscopic studies. There has been a rising need for efficient, accurate and less complex staining procedures. Many stain procedures are still in use today, and many others have been replaced with new immunostaining, molecular, non-culture and other advanced staining techniques. Some staining methods have been abandoned because the chemicals required have been medically proven to be toxic. The case studies indicated that in modern histology a combination of different stain techniques are used to enhance the effectiveness of the staining process. Currently, improved histological stains, have been modified and combined with other stains to improve their effectiveness.

Keywords: Molecular, microscopic, immunological, biological

Introduction:

Staining, in microbiology, can be defined as a technique which is used to enhance and contrast a biological specimen at the microscopic level. Stains and dyes are used to highlight the specimen at the microscopic level to study it at higher magnification for histopathological studies and diagnostic purposes [1].

However, staining is not just limited to biological specimens; it can also be used to study the structure of crystalline polymers.

Because microbial cytoplasm is usually transparent, it is necessary to stain microorganisms before they can be viewed with the light microscope [3]. In some cases, staining is unnecessary, for example when microorganisms are very large or when motility is to be studied, and a drop of the microorganisms can be placed directly on the slide and observed. A preparation such as this is called a wet mount. A wet mount can also be prepared by placing a drop of culture on a cover slip (a glass cover for a slide) and then inverting it over a hollowed-out slide. This procedure is called the hanging drop.

Smear Preparation

Not only are most bacteria very small, they are also very clear and difficult to view under a microscope without first staining. You must firmly attach your bacteria to a glass slide before you can stain them. There are two important things to consider when preparing a slide for staining:

1. The bacteria must be evenly and lightly dispersed. If there are too many bacteria on the slide they will form a big glob and you will not be able to see the morphology of the individual cells[4]. Large blobs of cells also do not stain properly and could yield erroneous results from the improper staining.
2. The bacteria need to be firmly attached to the slide so they are not washed off during the staining procedures. All procedures that attach the bacteria to the slide result in some morphological changes. The cells typically shrink in size and will exhibit some changes in shape and extra-cellular matrixes.

The simple stain

One type of staining procedure that can be used is the simple stain, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope. Some stains commonly used for simple staining include crystal violet, safranin, and methylene blue. Simple stains can be used to determine a bacterial species' morphology and arrangement, but they do not give any additional information [6]. Living bacteria are almost colorless, and do not present sufficient contrast with the water in which they are suspended to be clearly visible. The purpose of staining is to increase the contrast between the organisms and the background so that they are more readily seen in the light microscope. In a simple stain, a bacterial smear is stained with a solution of a single dye that stains all cells the same color without differentiation of cell types or structures. The single dye used here in our lab is methylene blue, a basic stain. Basic stains, having a positive charge, bind strongly to negatively charged cell components such as bacterial nucleic acids and cell walls.

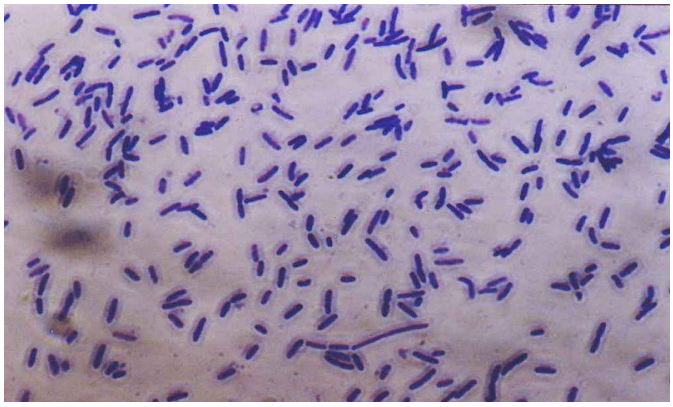

Figure 1: Simple Stain

Staining can be performed with basic dyes such as crystal violet or methylene blue, positively charged dyes that are attracted to the negatively charged materials of the microbial cytoplasm. Such a procedure is the simple stain procedure. An alternative is to use a dye such as nigrosin or Congo red, acidic, negatively charged dyes. They are repelled by the negatively charged cytoplasm and gather around the cells, leaving the cells clear and unstained. This technique is called the negative stain technique [8].

The gram stain

Scientists will often choose to perform a differential stain, as this allows them to gather additional information about the bacteria they are working with. Differential stains use more than

one stain, and cells will have a different appearance based on their chemical or structural properties. Some examples of differential stains are the Gram stain, acid-fast stain, and endospore stain. You will learn how to prepare bacterial cells for staining, and learn about the gram staining technique.

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decoloring process.

This very commonly used staining procedure was first developed by the Danish bacteriologist Hans Christian Gram in 1882 (published in 1884) while working with tissue samples from the lungs of patients who had died from pneumonia. Since then, the Gram stain procedure has been widely used by microbiologists everywhere to obtain important information about the bacterial species they are working with. Knowing the Gram reaction of a clinical isolate can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.

Gram stain results reflect differences in cell wall composition. Gram positive cells have thick layers of a peptidoglycan (a carbohydrate) in their cell walls; Gram negative bacteria have very little. Gram positive bacteria also have teichoic acids, whereas Gram negatives do not. Gram negative cells have an outer membrane that resembles the phospholipid bilayer of the cell membrane. The outer membrane contains lipopolysaccharides (LPS), which are released as endotoxins when Gram negative cells die. This can be of concern to a person with an infection caused by a gram negative organism.

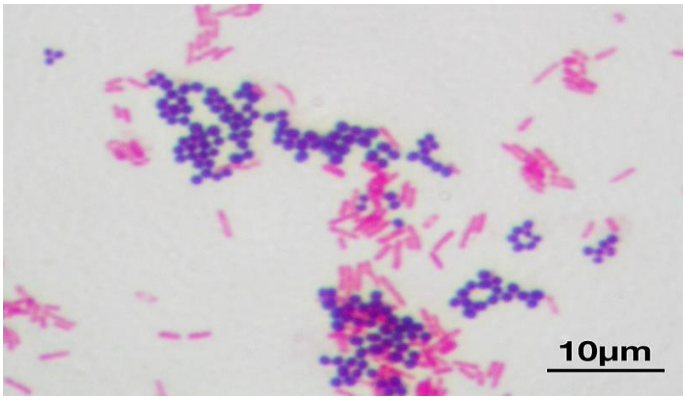

Figure 2: Gram Stain

The process involves three steps:

1. Cells are stained with crystal violet dye. Next, a Gram's iodine solution (iodine and potassium iodide) is added to form a complex between the crystal violet and iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water.

2. A decolorizer such as ethyl alcohol or acetone is added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. The large crystal violet-iodine complex is not able to penetrate this tightened peptidoglycan layer, and is thus trapped in the cell in Gram positive bacteria. Conversely, the the outer membrane of Gram negative bacteria is degraded and the thinner peptidoglycan layer of Gram negative cells is unable to retain the crystal violet-iodine complex and the color is lost.
3. A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram positive cells. However, the decolorized Gram negative cells are stained red.

Poor staining technique could lead to inaccurate results. One of the most important steps in Gram staining is the decolorizing step (use of alcohol/acetone). If the decolorizer is not left on long enough, then it will not be able to differentiate between Gram positive and Gram negative bacteria. This step uses decolorizer, made of an alcohol/acetone mixture. Its function in Gram negative bacteria is to remove the outer cell membrane and thin layer of peptidoglycan. The cell membrane is mostly made of lipids and are sensitive to alcohols. By dissolving these layers, the crystal violet-iodine complex is also removed, and thus Gram negatives are now able to take up the secondary stain, safranin, which is used in the last step of the Gram stain, staining them pinkish-red and differentiating between them and the Gram positives, who with their thick peptidoglycan layer has retained the primary stain, crystal violet, and appears purple/blue. On the flip side, if you use too much decolorizer, it can decolorize your sample on the slide, leading to loss of crystal violet (the primary stain)-iodine complex. The decolorizing step is sensitive because of the cell wall structure. Even Gram positive bacteria with their thick cell walls could become excessively decolorized, resulting in the loss of the peptidoglycan layer and the crystal violet-iodine complex. When the use of the secondary stain, safranin, is applied in the last step, the Gram positive bacteria will pick up this stain and look reddish-pink instead of purple/blue. Watch video 2 for an example of this.

Another common mistake is in the preparation of the bacterial smear, which is in the first step of any staining procedure. This involves applying a thin film of bacteria on your microscope slide and then heat fixing it with either your bunsen burner, bacticinerator, or slide warmer. The main purpose of this step is to adhere the bacterial cells to the microscope slide (it also denatures the proteins and kills them too). If you forget to do this step, then the cells will be 'washed' off in all the subsequent steps of your staining process. You will literally have no cells on your slide to stain!

Although the vast majority of bacteria are either Gram positive or Gram negative, it is important to remember that not all bacteria can be stained with this procedure (for example, Mycoplasmas, which have no cell wall, stain poorly with the Gram stain).

Differential stain techniques:

The differential stain technique distinguishes two kinds of organisms. An example is the Gram stain technique. This differential technique separates bacteria into two groups, Gram positive bacteria and Gram negative bacteria. Crystal violet is first applied, followed by the

mordant iodine, which fixes the stain. Then the slide is washed with alcohol, and the Gram positive bacteria retain the crystal violet iodine stain; however, the Gram negative bacteria lose the stain. The Gram negative bacteria subsequently stain with the safranin dye, the counterstain, used next. These bacteria appear red under the oil immersion lens, while Gram positive bacteria appear blue or purple, reflecting the crystal violet retained during the washing step.

Another differential stain technique is the acid fast technique. This technique differentiates species of *Mycobacterium* from other bacteria. Heat or a lipid solvent is used to carry the first stain, carbolfuchsin, into the cells. Then the cells are washed with a dilute acid alcohol solution. *Mycobacterium* species resist the effect of the acid-alcohol and retain the carbolfuchsin stain (bright red). Other bacteria lose the stain and take on the subsequent methylene blue stain (blue). Thus, the acid fast bacteria appear bright red, while the non-acid fast bacteria appear blue when observed under oil immersion microscopy.

Congo Red Capsule stain:

The Congo Red Capsule stain is a modification of the nigrosin negative stain you may have done previously. The bacteria take up the congo red dye and the background is stained then with acid fuchsin dye. The capsule or slime layers, highly hydrated polymers, exclude both dyes. The background will appear blue, the bacterial cells will appear pink, and the clear halos are the capsules.

Clinically, the capsules of some highly pathogenic bacteria (i.e.: pneumococci, *Haemophilis influenzae*, and meningococci), can be distinguished with the use of antisera specific for that type of capsule. The bacteria are suspended in the antisera and then mixed with methlyene blue. In the antisera staining procedure, the bacteria will appear blue surrounded by a clear halo and then surrounded by a thin blue line where the antisera have attached to the capsule.

Causes of poor quality of staining:

1. Poor or inadequate fixation of tissue.
2. Over or under-ripened Haematoxylin.
3. Overused or worked out Haematoxylin.
4. Over or under differentiation of haematoxylin
5. Insufficient blueing following differentiation.
6. Failure to wash blueing agent out of section before counter staining with eosin (especially when ammonia is used).
7. Insufficient differentiation of eosin during washing or dehydration.
8. Insufficient dehydration and clearing of sections.
9. Contamination of stains.

Conclusion:

Histological staining is a commonly used medical process in pathological diagnosis and forensic studies. The process of histological staining takes five key stages, and they include fixation, processing, embedding, sectioning and staining. Early histologists used the readily available chemicals to prepare tissues for microscopic studies; these laboratory chemicals were

potassium dichromate, alcohol and the mercuric chloride to hard cellular tissues. These fixatives and staining agents were ingenious and after a period colored staining agents were developed which are still applicable in the laboratory staining techniques today.Some staining methods have been abandoned because the chemicals required have been medically proven to be toxic. Similarly, there have been great changes in workload requiring more advanced technics of staining. The case studies indicate that, in the modern histology a combination of different stain techniques are used to enhance the effectiveness of the staining process. In the modern histologic as a way of improving histological stains, several stains have been modified and combined with other stains to improve their effectiveness.

References:

1. Anderson J. An introduction to Routine and special staining. 2011. [Retrieved on August 18, 2014 from. http://www.leicabiosystems.com/pathologyleaders/an-introduction-to-routine-and-special-staining .
2. Bancroft J. D, Layton C. The Hematoxylin and eosin. In: Suvarna S. K, Layton C, Bancroft J. D, editors. Theory Practice of histological techniques. 7th ed. Ch. 10 and 11. Philadelphia: Churchill Livingstone of El Sevier; 2013. pp. 179–220. http://dx.doi.org/10.1016/b978-0-7020-4226-3.00010-x .
3. Black J. Microbiology: Principles and exploration. 8th ed. John Wiley Sons; 2012. p. 68.
4. Cai H, Caswell J. L, Prescott J. F. (March 2014). Nonculture Molecular Techniques for Diagnosis of Bacterial Disease in Animals: A Diagnostic Laboratory Perspective. Veterinary Pathology. 51(2):341–350. http://dx.doi.org/10.1177/0300985813511132 .
5. Harris T. J, McCormick F. The molecular pathology of cancer. Nat Rev Clin Oncol. 2010;7(5):251–265. http://dx.doi.org/10.1038/nrclinonc.2010.41 .
6. Iyiola S, Avwioro O. G. Alum haematoxylin stain for the demonstration of nuclear and extra nuclear substances. Journal of Pharmacy and Clinical Sciences. 2011
7. Jackson P, Blythe D. Theory Practice of histological techniques. In SK. Suvarna, C. Layton JD. Bancroft (Eds. 7th ed. Ch. 18. Philadelphia: Churchill Livingstone of El Sevier; 2013. Immunohistochemical techniques; pp. 386–431. http://dx.doi.org/10.1016/b978-0-7020-4226-3.00018-4 .
8. Morelli P, Porazzi E, Ruspini M, Restelli U, Banfi G. Analysis of errors in histology by root cause analysis: a pilot study. J Prev Med Hyg. 2013;54:90–6.
9. Musumeci G. Past, present and future: overview on Histology and histopathology. J Histol Histopathol. 2014;1:5. http://dx.doi.org/10.7243/2055-091X-1-5 .
10. Nadworny P. L, Wang J. F, Tredget E. E, Robert E. Anti-inflammatory activity of nanocrystalline silver-derived solutions in porcine contact dermatitis. Burrel J. Inflammation. 2010. http://dx.doi.org/10.1186/1476-9255-7-13 .
11. Ntziachristos V. Going deeper than microscopy: the optical imaging frontier in biology. Nat Methods. 2010;7:603–614. http://dx.doi.org/10.1038/nmeth.1483 .

AN INTRODUCTION TO IMPACT OF MICROBIOLOGY TOWARDS BIOTECHNOLOGY

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Abstract:

The abstract provides an overview of the historical development of microbiology from its origins with the invention of lenses for visualizing microbes to modern genetic engineering. It emphasizes the importance of microbial classification based on various characteristics and highlights the significance of understanding bacterial cell structures and Gram staining technique. The abstract also discusses the role of microorganisms in food spoilage and preservation, as well as their application in bioremediation and biodeterioration prevention. Effective communication with stakeholders regarding microbial biotechnology's societal impacts is stressed. The diverse applications of microbiology in medical, agricultural, industrial, exo-microbiology, and geochemical fields are explored, encompassing the interactions between microorganisms and human well-being.

Keywords: Microscopy, microbiology, bacteria, gram staining, preservation, biodeterioration.

Introduction:

Historical facts:

Microscopy

Microbiology originated with the advent of lens grinding and assembly, allowing for the visualization of microbes due to enhanced magnification. In the thirteenth century, *Roger Bacon* (1220-1292) hypothesized that diseases could be caused by imperceptible living entities. This notion was later revisited by *Girolamo Fracastoro* of Verona (1483-1553) and *Anton von Plenciz* in 1782, but remained unsupported by evidence (Singer, 1914).

Around 1658, *Athanasius Kircher* (1601-1680), a monk, mentioned the existence of invisible worms in decaying bodies, meat, milk, and diarrheal secretions. In 1665, *Robert Hooke's* depiction of cells in cork samples reaffirmed that the complex bodies of animals and plants were composed of repeating elementary units, echoing the ancient concept described by *Aristotle* in the fourth century BCE (Reigoto, 2021).

Antony van Leeuwenhoek, living in Delft, Holland from 1632 to 1723, is credited with being the first to report his microscopic observations accurately and with detailed drawings. Despite not being the earliest observer of bacteria and protozoa, Leeuwenhoek's well-crafted microscopes, totalling over 250, made of home-ground lenses mounted in brass and silver, proved instrumental. The most powerful of these microscopes achieved magnifications of about 200 to 300 times, differing significantly from today's compound light microscopes capable of 1,000 to 3,000 times magnification. Nevertheless, Leeuwenhoek's lenses were of excellent

quality, and his inquisitive mindset significantly contributed to his success as an investigator. Many of the protozoa he accurately described during his time are still recognizable today (Singer, 1914; Chandra, 2017).

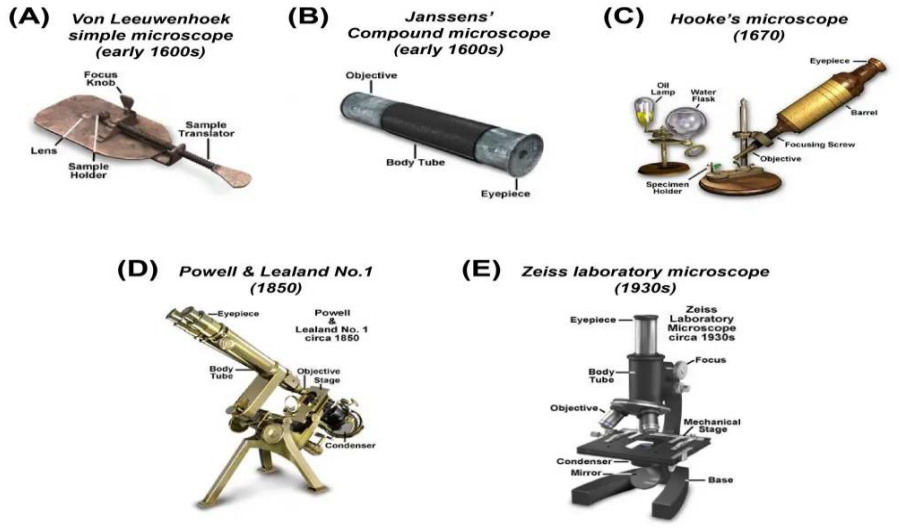

Figure 1: History of microscopes (Credits: rsscience.com)

Generational acceptance of biogenesis

The discovery of microbes instigated a profound fascination with the origin of living organisms, leading to a proliferation of debates and speculations. In the context of human beings, the Greek notion attributing the creation of people from stones and lifeless objects by the goddess Gaea had largely fallen out of favour. However, even the astute Aristotle (384-322 B.C.) postulated the possibility of animals spontaneously originating from soil, plants, or dissimilar animals, and his influence persisted well into the seventeenth century.

Presently, there is no known condition in which it can be affirmed that microscopic entities come into existence without the presence of germs or without parents of their own kind. Those who argue against this notion suggest that I have been deceived by illusions and flawed experiments, tainted by errors that were imperceptible and unavoidable (Reid, 1958).

Eventually, John Tyndall (1820-1883) conducted experiments within a specially designed box to demonstrate that dust carries germs. His experiments revealed that in the absence of dust, sterile broth remained free of microbial growth for indefinite periods.

Arouse of fermentation

Louis Pasteur initiated his illustrious career as a professor of chemistry at the University of Lille, France. Considering that the principal industry in France involved the production of wines and beer, Pasteur conducted an investigation into the methodologies and processes to aid his fellow citizens in achieving a consistently high-quality product. His research led him to discover that the fermentation of fruits and grains, yielding alcohol, was facilitated by microorganisms. Through the examination of numerous batches of ferment, he observed that specific types of microbes predominated in good lots, while other kinds were present in the inferior products. Pasteur proposed that by carefully selecting and controlling the microbes, manufacturers could ensure a uniform and excellent final product. He further suggested the

removal of undesirable microbial types through a gentle heating process, which would not adversely affect the flavour of the fruit juice but effectively diminish a significant portion of the microbial population. This involved holding the juices at a temperature of 62.8°C (145°F) for half an hour. Today, pasteurization has become extensively employed in fermentation industries, though it is most commonly associated with its application in the dairy industry (Chandra, 2017; Ali, 2021).

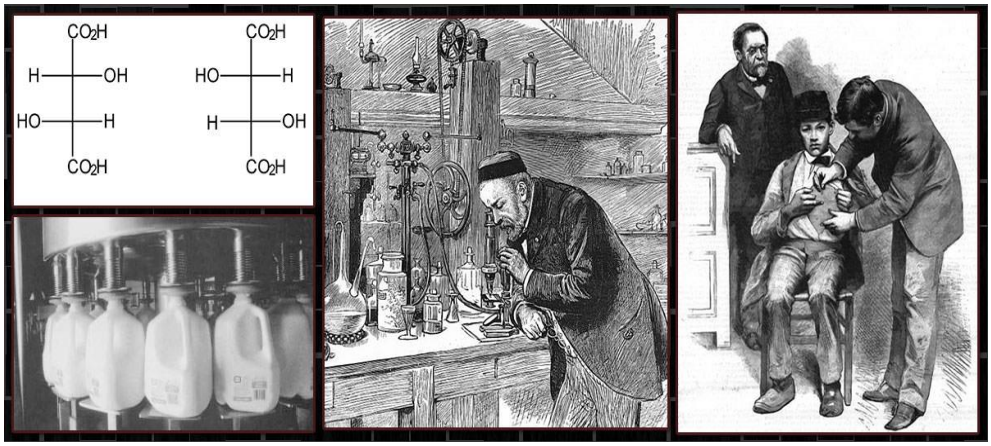

Figure 2: Pasteurization of milk and discovery of the first vaccine (Credits: learnodo-newtonic.com)

The gradual acceptance of microbiology

The foregoing account underscores the escalating significance of microbiology in modern society, with microbiology emerging as a paramount branch of the life sciences. Microbiologists have made substantial contributions to both fundamental biological sciences and various applied domains, encompassing public health, medical sciences, agriculture, industry, and environmental sciences. The most remarkable and current advancement in applied microbiology lies in the capacity to manipulate an organism's genetic composition, commonly known as genetic engineering. The comprehensive understanding of DNA's structure and function, coupled with the identification of enzymes that can "cut, unzip, or rebuild" the molecule, has facilitated the modification of microorganisms' DNA. The process of recombination enables the insertion of new DNA segments into the existing DNA, thus enabling the engineering of microorganisms through DNA modification.

Genetically modified microorganisms offer immense potential for the production of novel substances, such as human proteins. For instance, bacteria have been genetically engineered to synthesize human insulin and interferon. These genetically engineered microorganisms hold considerable promise in the realms of drug and vaccine production, enhancement of agricultural crops, and various other products and processes (Reid, 1958).

Microbial classification:

Major 8 classification points

The major characteristics of microorganisms can be classified into the following scientific categories (Al-mohanna, 2016):

Morphological characteristics: This category encompasses the cell's physical properties, including its shape, size, structure, arrangement, presence of specialized structures, developmental forms, staining reactions, motility, and flagellar arrangement.

Chemical composition: Refers to the various chemical constituents present within the cells, including molecules and compounds that make up their structure and play essential roles in their functions.

Cultural characteristics: Encompasses the nutritional requirements and physical conditions necessary for growth and the manner in which growth occurs, such as optimal temperature, pH, and availability of nutrients.

Metabolic characteristics: Pertains to how microorganisms obtain and utilize energy, the chemical reactions they perform, and the mechanisms they employ to regulate these reactions to sustain life processes.

Antigenic characteristics: Involves special large chemical components known as antigens that are specific to certain types of microorganisms, and they play a crucial role in immune responses and identification of these organisms.

Genetic characteristics: Focuses on the hereditary material of the cell, particularly DNA (deoxyribonucleic acid), and examines the presence and function of other kinds of DNA, such as plasmids, which may be present in microorganisms.

Pathogenicity: Describes the ability of microorganisms to cause disease in various hosts, including plants, animals, and other microorganisms.

Ecological characteristics: Investigates the habitat and distribution of microorganisms in nature, as well as the interactions that occur between and among different species in their natural environments (Ali, 2021).

In detailed explanation of the above characters is given below:

Morphological characters:

In contrast to other microbial traits, the determination of morphological characteristics typically necessitates the investigation of individual cells within a homogeneous culture. Microorganisms are diminutive entities, with their size commonly expressed in micrometres (µm). One micrometre corresponds to 0.001 millimetres (mm) or approximately 0.00004 inches. As a result, routine observation of microbial cells necessitates the utilization of a high-power microscope, typically operating at a magnification of around 1,000 times their original size. Employing electron microscopy allows for magnifications thousands of times and facilitates the visualization of intricate details of cell structure. Various techniques are accessible for the microscopic examination of microorganisms, with the chosen method contingent on the specific information sought (Al-mohanna, 2016).

Chemical characteristics:

Microbial cells display a diverse array of organic compounds. When cells are disassembled and their constituents subjected to chemical analysis, each type of microorganism exhibits a distinct chemical composition. Qualitative and quantitative variations in composition exist among different groups. For instance, the presence of lipopolysaccharide in cell walls is

characteristic of Gram-negative bacteria but not Gram-positive bacteria. Conversely, many Gram-positive bacteria possess cell walls containing teichoic acids, which are absent in Gram-negative bacteria. Fungal and algal cell walls differ significantly in composition from bacterial cell walls. Additionally, viruses are distinguished based on the type of nucleic acid they contain, either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) (Ali, 2021).

Cultural characteristics:

Each microorganism has specific growth requirements. Many can be cultivated in a culture medium, a laboratory mixture of nutrients that supports their growth and multiplication. Some microorganisms can thrive in a medium comprising only inorganic compounds, while others require organic compounds like amino acids, sugars, purines, pyrimidines, vitamins, or coenzymes. Some necessitate complex natural substances such as peptone, yeast autolysate, blood cells, or blood serum. Certain microorganisms cannot be grown in artificial laboratory media and can only propagate within a living host or living cells. For example, Rickettsia species require a host, such as an animal, fertilized chicken egg (chick embryo), arthropod, or culture of mammalian tissue cells, which serves as a complex "medium" for their nutrition (Al-mohanna, 2016)

Metabolic characteristics:

Microbial cell life processes involve a complex series of integrated chemical reactions collectively known as metabolism. The variety of these reactions provides opportunities to characterize and differentiate various microorganism groups. Different organisms may acquire energy through light absorption, oxidation of organic or inorganic compounds, or redistribution of atoms within certain molecules to increase instability. Organisms also differ in how they synthesize cell components during growth. Enzymes, specialized proteins, catalyse the various chemical reactions in an organism, and the complement of enzymes possessed by one type of organism, as well as their regulation, can vary significantly from other organisms (Ali, 2021).

Antigenic characteristics:

Specific chemical compounds of microbial cells are referred to as antigens. Antigenic characterization of microorganisms holds practical importance, as the animal body responds to these antigens by producing specific blood serum proteins called antibodies, which bind to the antigens. Antibodies are highly specific for the inducing antigens, and they are used as tools for rapid identification of particular microorganisms. This process can be likened to a "lock and key system," where the specificity of the reaction allows for identifying unknown microorganisms by testing their reaction with known antibodies. Antigenic characteristics aid in identifying different types of microorganisms (Ali, 2021).

Genetic characteristics:

Each type of microorganism's double-stranded chromosomal DNA exhibits constant and characteristic features useful for classification. Two main genetic characteristics include:

DNA base composition: DNA consists of base pairs guanine-cytosine and adenine-thymine. The percentage of guanine plus cytosine relative to the total nucleotide bases is referred to as the moles % G+C value, which varies between 23 to 75 for different organisms. Sequence of

nucleotide bases in the DNA: This sequence is unique to each organism and is fundamental for microbial classification (Ali, 2021).

Additionally, plasmid DNA may be present in microbial cells, which are circular DNA molecules capable of autonomous replication within bacteria. Plasmids can confer special characteristics on the host cells, such as toxin production, antibiotic resistance, or the ability to use unusual chemical compounds as nutrients.

Pathogenicity:

The ability of some microorganisms to cause disease, known as pathogenicity, is a prominent characteristic that has driven much early research with microorganisms. While only a relatively small number of species cause disease, certain microorganisms are pathogenic for animals or plants, and some may infect and harm other microorganisms, such as predatory bacteria and bacteriophages that target bacterial cells (Ali, 2021).

Ecological characteristics:

The habitat of a microorganism plays a significant role in characterizing the organism. Microorganisms found in marine environments differ from those in freshwater settings. The microbial population of the oral cavity varies from that of the intestinal tract. Some microorganisms are widespread in nature, while others are restricted to specific environments. The relationship between an organism and its environment can be intricate and may involve yet unknown special characteristics of the organism (Ali, 2021).

Identification of bacterial cells

Size, shape and arrangement:

Bacteria, which are microorganisms, typically exhibit a size ranging from approximately 0.5 to 10 µm in diameter. One significant implication of their small size is the remarkably high surface area/volume ratio in comparison to larger organisms with similar shapes (Table 51). This high ratio allows for a relatively large surface area for nutrient intake and waste product release, while the volume of cell substance that needs nourishment remains small. Consequently, bacteria can achieve an exceptionally rapid rate of growth and metabolism. The proximity of the cell substance to the surface due to the high surface area/volume ratio eliminates the need for a circulatory mechanism to distribute nutrients within bacterial cells. As a result, there is little or no cytoplasmic movement in bacteria. Despite these advantages, the limitation imposed by a high surface area/volume ratio confines bacteria to microscopic dimensions (Al-mohanna, 2016).

Bacterial shape and arrangement are influenced by their rigid cell walls, although the specific attributes determining a cell's shape are not yet fully understood. Typical bacterial cells are found to be spherical (cocci, singular coccus), straight rods (bacilli, singular bacillus), or helically curved rods (spirilla, singular spirillum). While most bacterial species exhibit fairly constant and characteristic shapes, some can display pleomorphism, meaning they can take on a variety of shapes (Al-mohanna, 2016).

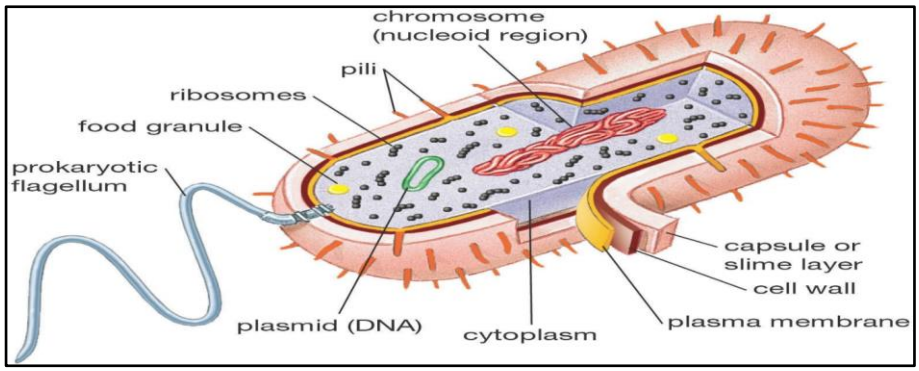

Figure 3: Structure of bacterial cell (Credits: 1.bp.blogspot.com)

Flagella:

Among the common structures in bacteria, flagella are hair-like, helical appendages that extend through the cell wall and are responsible for the bacterium's swimming motility. These flagella are much thinner than those found in eukaryotes, with a diameter of 0.01 to 0.02 µm, and they possess a simpler structure. The location of flagella on the bacterial cell depends on the species and can be polar (at one or both ends of the bacterium) or lateral (along the sides of the bacterium). In some Gram-negative bacteria, the flagellum is surrounded by a sheath continuous with the outer membrane of the cell wall. The chemical composition of the basal body is still unknown, but the hook and filament are composed of protein subunits (monomers) arranged in a helical manner, with the filament's protein known as flagellin.

Notably, flagella grow at their tips rather than their bases, and flagellin monomers synthesized within the cell are believed to travel along the hollow centre of the flagellum and be added to the distal end of the filament (Al-mohanna, 2016).

Pili (Fimbriae):

Unlike flagella, they do not function in motility and can be found on both motile and non-motile bacterial species. Different types of pili serve various functions. For instance, the F pilus (or sex pilus) acts as the entry port for genetic material during bacterial mating. Some pili play a significant role in human infection by facilitating the attachment of pathogenic bacteria to epithelial cells lining the respiratory, intestinal, or genitourinary tracts. This attachment prevents the bacteria from being washed away by mucous or body fluids, enabling the establishment of infection (Ali, 2021).

Capsules:

This layer is visible under light microscopy using specific staining methods, it is referred to as a capsule. If the layer is too thin to be observed with light microscopy, it is called a microcapsule. When the material is abundant enough for many cells to be embedded in a common matrix, it is termed slime.

Capsules appear as amorphous gelatinous areas surrounding the bacterial cell under light microscopy. However, advanced electron microscopy techniques have revealed that capsules consist of a mesh or network of fine strands. In many cases, the capsular material is not highly water-soluble, making it less prone to diffuse away from the cells that produce it. On the other

hand, some capsules are highly water-soluble and dissolve in the medium, sometimes leading to a significant increase in the viscosity of the culture broth (Al-mohanna, 2016).

Sheaths:

In various bacterial species, especially those thriving in freshwater and marine ecosystems, a fascinating phenomenon occurs where they assemble into chains or trichomes. These chains are enveloped by a hollow tube-like structure called a "sheath." This sheath becomes more visibly discernible when certain cells have separated or migrated from it. What's even more intriguing is that these sheaths have the potential to undergo a transformation. At times, they become infused with ferric or manganese hydroxide, resulting in an augmentation of their structural robustness. This adaptation likely plays a role in the survival and resilience of these bacteria in their respective aquatic habitats. The impregnation of minerals adds an extra layer of protection, granting them the strength to endure environmental challenges and fluctuations.

Overall, this remarkable interplay of bacterial organization and the sheath's unique properties offers valuable insights into the adaptability and survivability of certain bacteria in freshwater and marine environments. It also underscores the significance of understanding microbial interactions within natural ecosystems to comprehend the intricate dynamics that shape life on our planet.

The cell wall:

Located beneath external structures such as capsules, sheaths, and flagella, and external to the cytoplasmic membrane, is the cell wall—a highly rigid structure responsible for providing shape to the bacterial cell. Its primary function is to prevent the cell from expanding and potentially rupturing due to water uptake, especially since most bacteria inhabit hypotonic environments (i.e., environments with lower osmotic pressure than the bacterial cells' internal environment). The cell wall's rigidity is readily demonstrated by subjecting bacteria to high pressures or severe physical conditions, as most bacterial cells retain their original shapes during and after such treatments. To analyse isolated cell walls, bacteria typically need to be mechanically disintegrated through drastic means, such as sonic or ultrasonic treatment or exposure to extremely high pressures followed by sudden release. The broken cell walls are then separated from the rest of the disintegrated cell components through differential centrifugation, retaining the original shape of the cells from which they were derived (Al-mohanna, 2016).

Differentiation of bacterial cell walls depending on their gram nature:

Gram-positive cell walls:

The gram-positive cell walls contain substantial amounts of teichoic acids, which are glycerol or ribitol polymers connected by phosphate groups. These teichoic acids may have amino acids like D-alanine or sugars like glucose attached to them. The teichoic acids can be covalently bonded to the peptidoglycan itself or to plasma membrane lipids, in which case they are referred to as lipoteichoic acids. The negatively charged teichoic acids extend to the surface of the peptidoglycan, contributing to the gram-positive cell wall's overall negative charge. While the exact functions of teichoic acids are not entirely understood, they are believed to play a role

in maintaining the wall's structure. It's important to note that gram-negative bacteria do not possess teichoic acids (Beveridge, 1990).

Gram-negative cell walls:

Gram-negative cell walls are significantly more complex than gram-positive cell walls. The thin peptidoglycan layer adjacent to the plasma membrane constitutes only a small portion (5 to 10%) of the wall's weight. In certain bacteria like E. coli, the peptidoglycan layer can be approximately 2 nm thick and comprise only one or two layers of peptidoglycan sheets. In some cases, the peptidoglycan may be in the form of a gel rather than a compact layer. The outer membrane lies outside this thin peptidoglycan layer. Braun's lipoprotein, a small lipoprotein covalently attached to the underlying peptidoglycan and embedded in the outer membrane through its hydrophobic end, is the most abundant membrane protein. The outer membrane and peptidoglycan are firmly linked through this lipoprotein, allowing them to be isolated as one unit. Another structure, the adhesion site, may further strengthen the gram-negative wall and hold the outer membrane in place. Adhesion sites are regions of direct contact or possible membrane fusions between the outer membrane and plasma membrane, seen as 20 to 100 nm areas of contact in plasmolyzed E. coli cells. These sites could potentially allow substances to move into the cell without traversing the periplasm (Beveridge, 1990).

One of the most distinct components of the outer membrane is lipopolysaccharide (LPS). LPS is a large, complex molecule containing both lipid and carbohydrate regions, composed of three parts: (1) lipid A, (2) the core polysaccharide, and (3) the O side chain. Lipid A, buried in the outer membrane, contains glucosamine sugar derivatives, each with three fatty acids and attached phosphate or pyrophosphate groups. The rest of the LPS molecule projects from the bacterial surface. The core polysaccharide, attached to lipid A, consists of 10 sugars with unusual structures in Salmonella. The O side chain, a short polysaccharide chain, extends outward from the core and varies in composition between bacterial strains. The O side chains are recognized by host antibodies, but gram-negative bacteria may alter their O side chains to evade host defences. Apart from evading host defences, LPS contributes to the negative charge on the bacterial surface, stabilizes membrane structure, and acts as an endotoxin, causing symptoms in gram-negative bacterial infections. The outer membrane serves as a protective barrier, preventing or slowing the entry of bile salts, antibiotics, and other toxic substances that could harm the bacterium. Despite its protective function, the outer membrane is more permeable than the plasma membrane, allowing the passage of small molecules like glucose through special porin proteins. These porin molecules cluster together and form a narrow channel through which small molecules can pass. Larger molecules like vitamin B12, however, require specific carriers to transport them across the outer membrane (Sandle, 2004).

Elucidating the mechanism of gram staining in bacterial cell wall:

The Gram staining method is widely used to differentiate between gram-positive and gram-negative bacteria. Despite various proposed explanations for the differential staining, it appears that the key factor lies in the physical properties of their respective cell walls. This study explores the mechanism of Gram staining, focusing on the role of peptidoglycan, a major

component of bacterial cell walls. We demonstrate that peptidoglycan acts as a permeability barrier, preventing the loss of crystal violet during the staining process. Additionally, we investigate the impact of alcohol treatment on the cell walls, revealing its ability to alter the porosity of gram-positive and gram-negative peptidoglycan. Our findings shed light on the factors responsible for the differential staining and provide insights into the structural aspects of bacterial cell walls that underlie the Gram staining technique. (Sandle, 2004)

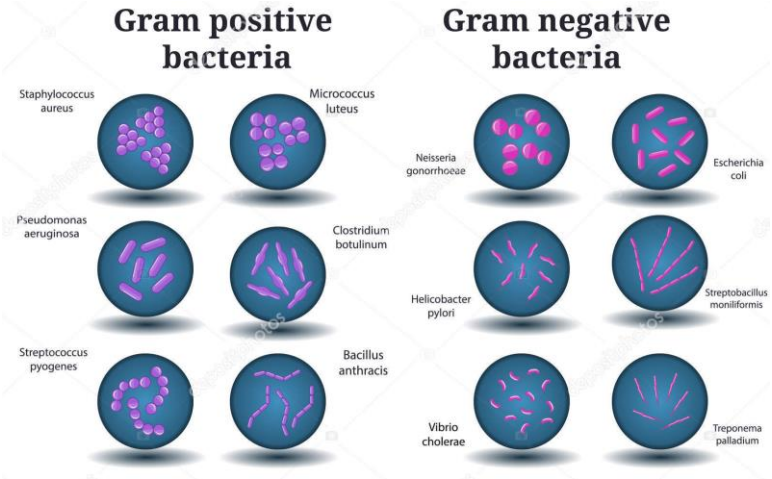

Figure 4: Gram positive and gram negative bacterial classification (Credits: st4.depositphotos.com)

Role of microbes in spoilage and preservation

Microorganisms and their impact on food spoilage:

The shift from hunting and gathering to agriculture brought about the need to preserve surplus foods for survival. In ancient Near Eastern civilization, around 3000 B.C., methods such as using salt as a meat preservative and producing cheeses and curdled milks were introduced. The preservation of fish and meat by smoking, as well as the production of wines, also became common practices during this time. However, despite early preservation efforts, it was not until the nineteenth century that the study of microbial spoilage of food was undertaken in a rigorous manner. In 1857, Louis Pasteur played a significant role in establishing the modern era of food microbiology by demonstrating that microorganisms were responsible for milk spoilage. His subsequent work in the 1860s further confirmed that heat treatment could be employed to control spoilage organisms in wines and beers (Sevindik, 2021).

Various intrinsic and extrinsic factors determine whether microbial growth will preserve or spoil foods, as depicted in figure. On the other hand, extrinsic factors are related to the food's external environment and encompass parameters such as temperature, relative humidity, and the types and numbers of microorganisms present in the food, as well as the gases (CO_2, O2) present in the surrounding environment. It is mainly included in two main factors i.e., intrinsic and extrinsic which are explained in detail below (Technology, 2020).

Intrinsic factors:

Food composition represents a critical intrinsic determinant that impacts the spoilage process. Foods primarily composed of carbohydrates exhibit minimal odour during spoilage, and the initial signs of spoilage are manifested through fungal growth, as observed in breads, jams,

and certain fruits. In contrast, foods rich in proteins and/or fats, such as meat and butter, undergo spoilage accompanied by a diverse range of unpleasant odours, akin to the smell of rotting eggs. This process, known as putrefaction, involves the anaerobic breakdown of proteins, leading to the production of foul-smelling amine compounds like cadaverine. Additionally, fats' degradation results in food spoilage, exemplified by the rancidity of butter due to the production of short-chained fatty acids.

The pH level of a food significantly influences spoilage, with low pH favouring the growth of yeasts and molds, while neutral or alkaline pH promotes bacterial dominance in the spoilage and putrefaction processes. The type of spoilage that occurs is contingent on the predominant substrate present in the food.

Moreover, the presence and availability of water influence microorganisms' ability to colonize foods. By reducing the water content through drying, one can control or eliminate spoilage processes. Solutes like sugar and salt can be added to lower the availability of water, measured in terms of water activity (aw). High concentrations of salt or sugar can dehydrate most microorganisms due to hypertonic conditions, preventing their growth. However, certain osmophilic and xerophilic microorganisms may still cause food spoilage under adverse conditions. Osmophilic microorganisms thrive in high osmotic concentration environments, while xerophilic microorganisms prefer low aw conditions and may not grow under high aw conditions (Sevindik, 2021).

The oxidation-reduction potential of a food also plays a role in spoilage. Cooked meat products, particularly broths, often have lower oxidation-reduction potentials, creating an ideal medium for the growth of anaerobic microorganisms, including Clostridium.

Additionally, the physical structure of a food can affects the extent and pace of spoilage. Grinding and mixing of foods like sausage and hamburger increase the food surface area, alter cellular structure, and distribute contaminating microorganisms throughout the food, leading to rapid spoilage if stored improperly. Vegetables and fruits possess outer skins (peels and rinds) that protect them from spoilage, but specialized enzymes in spoilage microorganisms can weaken and penetrate these protective layers, particularly after the fruits and vegetables have been bruised. For instance, coumarins in fruits and vegetables exhibit antimicrobial activity. Cow's milk and eggs also contain antimicrobial substances, such as lysozyme, which can lyse the cell walls of contaminating gram-positive bacteria. Some hot sauces, like Tabasco, demonstrate desirable antimicrobial characteristics when used with raw oysters and seafood. Herbs and spices often possess significant antimicrobial substances as well, with fungi being more sensitive than most bacteria. For example, sage and rosemary are two spices with strong antimicrobial properties. Aldehydic and phenolic compounds found in cinnamon, mustard, and oregano inhibit microbial growth. However, it is important to note that spices can also carry pathogenic and spoilage organisms, necessitating proper sterilization methods (Sevindik, 2021).

Unfermented green and black teas are known for their antimicrobial properties due to their polyphenol content, but these properties may diminish during fermentation.

Extrinsic factors:

Temperature and relative humidity represent important extrinsic factors that influence food spoilage. Higher relative humidities lead to more rapid microbial growth, even at lower temperatures (especially when refrigerators are not defrosted properly). When drier foods are exposed to moist environments, moisture absorption occurs on the food surface, creating conditions conducive to microbial growth (Technology, 2020).

The storage atmosphere is another crucial factor. For shrink-packed foods, some plastic films allow oxygen diffusion, resulting in increased growth of surface-associated microorganisms. An excess of carbon dioxide (CO_2) can decrease the solution pH, inhibiting microbial growth. Storing meat in a high CO2 atmosphere inhibits gram-negative bacteria, leading to a population dominated by lactobacilli (Sevindik, 2021).

Food preservation alternatives:

Various techniques can be employed for food preservation, and it is crucial to eradicate or decrease the presence of spoilage and pathogenic microorganisms while maintaining the microbiological quality of food through proper storage and packaging. Contamination often occurs after the opening of a package or can, just before the food is served, providing an ideal environment for pathogen growth and transmission if precautions are not taken. Utilizing chemical sanitizers like chlorine or quaternary ammonium compounds for washing utensils and limiting human contact with food significantly impact the microbiological quality of foods (Technology, 2020).

Filtration

Microorganisms in water, wine, beer, juices, soft drinks, and other liquids can be removed through filtration, effectively reducing or eliminating bacterial populations. Prefilters and centrifugation are often used to enhance filter lifespan and efficiency. Some beer brands prefer filtration over pasteurization to preserve the original product's flavour and aroma (Kumawat, 2020).

Low or high temperature

Refrigeration at 5°C retards microbial growth, but extended storage can still lead to growth and spoilage by psychrophiles and psychrotrophs. Some microorganisms are sensitive to cold, leading to reduced numbers, but overall microbial populations do not significantly decrease. Utilizing high temperatures for food can control microbial populations, limiting disease transmission and spoilage. Pasteurization involves high temperatures to eliminate disease-causing organisms and reduce microbial populations, while sterilization uses high temperatures to eliminate all living organisms. Heating processes, as developed by Nicholas Appert in 1809, offer a safe means of food preservation, especially in commercial canning operations. Canned food is heated in retorts at approximately 115°C for varying intervals based on the food type. Sometimes canning may not kill all microorganisms but eliminates those that would cause spoilage, with remaining bacteria unable to grow due to food acidity (Torres-León, 2022).

Pasteurization involves heating food to a specific temperature for a particular time to reduce disease-causing microorganisms and spoilage organisms. Dehydration, including freeze-

drying, is a common method for eliminating microbial growth by removing free water and increasing solute concentration in the remaining water (Technology, 2020).

Chemicals and radiation

These agents, such as organic acids, sulphite, ethylene oxide, sodium nitrite, and ethyl formate, disrupt critical cell factors in microorganisms, damaging plasma membranes, denaturing cell proteins, or interfering with nucleic acid functioning to inhibit cell reproduction. The effectiveness of chemical preservatives depends on the food's pH, with some compounds being more effective at lower pH values. For instance, sodium propionate is commonly used as a preservative in low pH bread products. Sodium nitrite plays a significant role in preserving cured meats by inhibiting Clostridium botulinum growth and spore germination, preventing botulism and reducing spoilage rates. (Kumawat, 2020)

Figure 5: Preservative methods (Credits: www.teachoo.com)

Biodegradation and its management

Biodegradation is a broad term that encompasses the microbial-mediated decomposition of various materials, such as paper, paint, textiles, concrete, hydrocarbons, and others. It involves three distinct definitions, namely minor molecular changes, fragmentation, or complete mineralization, as described in chapter 42. Sometimes it is necessary to enhance degradation processes, as seen in bioremediation, which utilizes microorganisms to eliminate pollutants from the environment. This can be achieved by intentionally stimulating biodegradation through engineered bioremediation or allowing natural biodegradation to occur under intrinsic bioremediation conditions.

Stimulation of biodegradation

Early efforts to stimulate microbial activities involved modifying water and soil environments through the addition of oxygen or nutrients, now known as engineered bioremediation. The success of this managed process depends on critical factors such as contact between microbes and the substrate, appropriate physical conditions, availability of nutrients, presence of oxygen (in most cases), and absence of toxic compounds. Oil spills in marine environments serve as a practical illustration of these principles. For instance, when dealing with

dispersed hydrocarbons in the ocean, it is crucial to maintain contact between microorganisms, the hydrocarbon substrate, and other essential nutrients. This has been achieved through the use of nutrient-containing pellets combined with an oleophilic (hydrocarbon soluble) preparation. This technique has resulted in a 30 to 40% acceleration in the degradation of various crude oil slicks compared to control oil slicks without additional nutrients.

A notable challenge for this technology arose from the Exxon Valdez oil spill in Alaska in March 1989. Different approaches were employed to enhance biodegradation, including nutrient additions, chemical dispersants, biosurfactant additions, and high-pressure steam utilization. The application of a glycolipid emulsifier produced by microorganisms proved beneficial. However, high-temperature steam caused significant disruption to the microbial community. In laboratory and field studies, attempts have been made to expedite biodegradation by introducing known degradative microorganisms. These microbes have been isolated from contaminated field sites, obtained from culture collections, or even genetically modified to enhance their degradative capabilities.

Control of biodeterioration

While biodegradation is desirable in many cases, it can also lead to severe problems when it inadvertently results in the destruction or damage of valuable materials. Several examples highlight the importance of addressing this issue and implementing control measures (Narancic, 2018).

Jet fuels

Early challenges related to microbial-induced changes in jet fuels involved the growth of microorganisms at the interface between fuel and accumulated trace amounts of water in storage facilities and airplane fuel tanks. Microorganisms, particularly Cladosporium resinae, commonly known as the kerosene fungus, caused clogging of critical pumps and orifices. To control growth at the jet fuel/water interface, various microbial inhibitors such as organoboron and isothiazolone compounds have been employed. For instance, a mixture of methylchloro/methyl-isothiazolone prevents fungal growth at a concentration of 1 ppm. Cleaning fuel storage and aircraft fuel tanks more frequently, along with the use of filters in fuel delivery and transfer lines, has also been effective in combating the problem (Levy, 2004).

Paper

Microbial degradation of stored softwoods used in paper manufacturing can lead to significant economic losses. The growth of microorganisms decreases paper strength, causes discoloration, and accelerates the deterioration of the final paper product. Papermaking involves the chemical solubilization of lignin and hemicellulose, releasing wood sugars that microorganisms can utilize. These soluble waste products are removed from the residual cellulose through extensive washing. However, microorganisms present in the wash solutions produce slimes and surface growths, which reduce process efficiency, final paper yield, and quality. In the past, mercury-containing organic and inorganic compounds were commonly used as biocides in pulp and paper manufacturing plants, with long-term effects on lakes and rivers in timber-producing regions. Nowadays, other compounds such as chlorine, phenols, and

organosulfide compounds are employed as biocides. Extensive washing with hot alkali is often employed to maintain slime-free conditions in papermaking equipment (Mahdy, 2016).

Computer chips

Microbial contamination poses a significant concern in the computer chip and electronics industries. It can decrease the service life of transistors by impairing the adhesion of successive coatings during chip manufacturing. Microbial growth at transistor junctions creates serious problems in critical electronic components. Therefore, the use of ultrapure water, devoid of dissolved organic matter and microorganisms, is essential in modern electronics manufacturing. Even with meticulous membrane filtration, the presence of minicells can result in microbial contaminants, even in water passed through 0.22 μm membrane filters. This is due to the ability of microorganisms to grow using low levels of organic compounds leached from plastic tubing or absorbed from the air (Jung, 2015; Mahdy, 2016).

Textiles and leather

Degradation of cloth and leather materials, particularly in tropical or humid environments, presents an ongoing economic problem. To mitigate microbial degradation, fungal inhibitors, including various phenolic-based compounds, can be added to natural materials like cotton and wool. Additionally, the use of copper compounds in tanning processes enhances the resistance of leather products to microbiological degradation (Egan, 2022).

Impacts of microbial biotechnology:

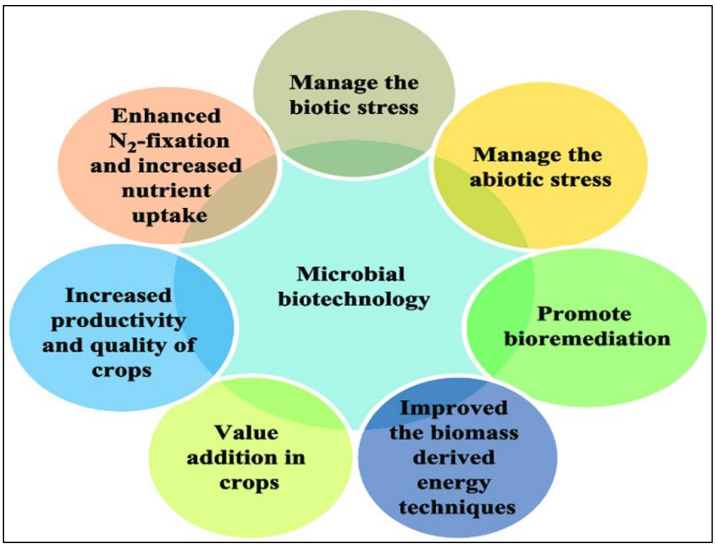

Figure 6: Impacts of microbial biotechnology (Credits: www.researchgate.com)

The impacts of microbial biotechnology, as explored in this chapter, do not occur in an isolated ethical and ecological context. The decisions made regarding the development and application of specific products and methodologies can lead to enduring and unforeseen consequences, exemplified by the emergence of antibiotic-resistant pathogens globally. For any microbiologist engaged in biotechnology fields, it is imperative to carefully contemplate the long-term ramifications of their technical choices. A comprehensive understanding of the potential societal impacts of novel products and processes, both within the realm of microbiology and beyond, is the primary challenge faced by microbiologists. To fulfil this responsibility,

effective communication with various "societal stakeholders" about the immediate and long-term implications of microbial-based (and other) technologies is essential. Samuel Floorman's work provides a valuable introduction to the relationship between technology and its potential societal impacts (Anquan and Prasad, 2020).

Scope in microbiology

Heterogenous nature of microorganisms:

Microorganisms exhibit ubiquitous occurrences in natural environments. They are disseminated through aerial currents, facilitating their transportation from terrestrial surfaces to the upper atmosphere. Even indigenous oceanic microorganisms can be detected miles away, atop mountainous regions. Profound oceanic depths harbour microbial populations, while fertile soil abounds with their abundance. Streams and rivers serve as conduits for their transfer into lakes and other substantial water bodies, potentially facilitating the spread of diseases when human waste carrying harmful bacteria is discharged into such water systems (Singh, 2020).

Microbial proliferation predominantly transpires where conducive conditions for sustenance and multiplication, including the presence of food, moisture, and suitable temperatures, are met. Given that these conditions align with the typical human living environment, it becomes inevitable for us to coexist amidst a myriad of microorganisms. Microbes are pervasive in the air we breathe, the food we consume, and are omnipresent on our body surfaces, including alimentary tracts, oral and nasal cavities, and other bodily orifices. Fortunately, the majority of microorganisms pose no harm to human health, and we possess mechanisms to counteract potential invasions by those that are pathogenic (Singh, 2020).

Applied areas in microbiology:

Applied areas of microbiology encompass a wide array of interactions between microorganisms and human well-being. Microorganisms thrive abundantly in various natural habitats, manifesting both beneficial and detrimental effects. Their activities encompass an extensive spectrum, including pathogenicity towards humans, animals, and plants, as well as mineral production, coal formation, and soil fertility enhancement. It is worth noting that the number of microorganism species contributing to essential ecological functions in nature far exceeds those responsible for causing diseases. The table below provides an overview of the principal fields of applied microbiology (Darvishi, 2014).

Sr. No.	Applied Areas	General Information
1.	Medical microbiology	Investigates the etiological agents of diseases and utilizes diagnostic methodologies to identify these causative agents. Additionally, preventive measures are studied and implemented to control disease transmission.
2.	Aquatic microbiology	Focuses on water purification and employs microbiological examinations to monitor water quality. Moreover, the field explores biological processes involved in waste degradation and examines aquatic ecosystems' ecology.

3.	Aero microbiology	It is concerned with the contamination and spoilage of air by microorganisms, as well as the dissemination of airborne diseases.
4.	Food microbiology	It centres around food preservation and preparation techniques, while also studying foodborne diseases and strategies for their prevention.
5.	Agricultural microbiology	Mainly addresses soil fertility and investigates diseases affecting both plants and animals in agricultural settings.
6.	Industrial microbiology	It encompasses the production of medicinal products, such as antibiotics and vaccines, along with the fermentation of beverages and industrial chemicals. It also involves the synthesis of proteins and hormones using genetically engineered microorganisms.
7.	Exo-microbiology	Involves the search for life in outer space, exploring the possibility of microbial existence beyond Earth.
8.	Geochemical microbiology	It explores microbial involvement in coal, mineral, and gas formation, and aids in prospecting for coal, oil, and gas deposits. Additionally, it contributes to the recovery of minerals from low-grade ores.

References:

1. Ali Jabber Al-Maliki (2021). Classification of Microbes, Missan University
2. Ali Jabber Al-Maliki (2021). Bacterial Cell Structures and Functions, Missan University.
3. Moshtaq Talip Al-mohanna (2016). Bacterial introduction, University of Al-Qadisiyah
4. Augustin M.A., L. Sanguansri, E.M. Fox, L. Cobiac and M.B. Cole (2020). Recovery of wasted fruit and vegetables for improving sustainable diets, Trends in Food Science and Technology, 95 (2020), pp. 75-85.
5. Beveridge, T. J. (1990). Mechanism of gram variability in select bacteria. J. Bacteriol., 172, 1609–1620. doi: 10.1128/jb.172.3.1609-1620.1990.
6. Chandra, Shaneel and Chapman, James and Power, Aoife and Roberts, Jess and Cozzolino, Daniel. (2017). Fermentation.
7. Darvishi, F. (2014). Microbial Biotechnology: Progress and Trends.10.13140/2.1.3187.6804.
8. Egan, J., Salmon, S. (2022). Strategies and progress in synthetic textile fiber biodegradability. SN Appl. Sci. 4, 22 (2022). https://doi.org/10.1007/s42452-021-04851-7.
9. Jung, Y., Chang, TH., Zhang, H. (2015). High-performance green flexible electronics based on biodegradable cellulose nanofibril paper. Nat Commun 6, 7170 (2015).
10. Kumawat, D. (2020). Food preservation. food preservation science.
11. Levy, J. A. (2004). Directory of Microbicides for the Protection of Materials. 2004;pp 177 DOI: 10.1007/1-4020-2818-0_1.

12. Mahdy, Dhuha and And, Jabir and Jabir, Mustafa (2016). A study of biodegradation of paper wastes by using bacteria isolated from the soil. 10.13140/RG.2.2.33601.97124.
13. Narancic T., Verstichel S., Reddy Chaganti S., Morales-Gamez L., Kenny S.T., De Wilde B., Babu Padamati R., O'Connor K.E. (2018). Biodegradable Plastic Blends Create New Possibilities for End-of-Life Management of Plastics.
14. Pitt TL, Barer MR. (2012). Classification, identification and typing of micro-organisms. Medical Microbiology. 2012:24–38. doi: 10.1016/B978-0-7020-4089-4.00018-4. Epub 2012 May 24. PMCID: PMC7171901.
15. Reigoto AM, Andrade SA, Seixas MCRR, Costa ML, Mermelstein C (2021). A comparative study on the use of microscopy in pharmacology and cell biology research. PLoS ONE 16(1): e0245795.
16. Roger Delbert Reid Krishna Prakashan Media, Michael Joseph Pelczar. Bacteriology. Michael Joseph Pelczar, Roger Delbert Reid Krishna Prakashan Media, 1958 - Bacteriology - 564 pages, 1958.
17. Sandle, T. (2004). Gram's Stain: History and Explanation of the Fundamental Technique of Determinative Bacteriology. Institute of Science Technology Journal.
18. Sevindik, Mustafa and Uysal, İmran (2021). Food spoilage and Microorganisms. Turkish Journal of Agriculture, 9: 1921-1924. 10.24925/turjaf.v9i10.1921-1924.4658.
19. Shanquan and Prasad, Ram (2020). Microbial Biotechnology: Basic Research and Applications. 10.1007/978-981-15-2817-0.
20. Singer. (1914). Notes on the Early History of Microscopy. Proc R Soc Med. 1914;7(Sect Hist Med):247-79.
21. Singh, Joginder and Vyas, Ashish and Wang, Shanquan and Prasad, Ram (2020). Microbial Biotechnology: Basic Research and Applications. 10.1007/978-981-15-2817-0.
22. Torres-León, Cristian and Aguilar, Cristobal (2022). Food Preservation. 10.1201/9781003277453-4.

NANOTECHNOLOGY IN MANAGEMENT OF NEMATODE PESTS

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Abstract:

Plant parasitic nematodes (PPNs) have emerged as a severe threat to crop production and are responsible for an estimated loss of US $173 billion annually to world agriculture. In India they are reported to cause an average crop loss of 21.3% annually. Use of chemicals provides immediate effect to prevent pest population but also, it is estimated that 90% of applied pesticides are lost during or after application which leads to the contamination of environment. The cultural and biological approaches give slow results and sometimes are not even viable for intensive commercial agriculture. Nanotechnology is the science that deals with synthesis and application of nano sized particles (1-100 nm or 1.0 ×10-9m) of any material. The nanoparticles (NP's) have a high surface to volume ratio that increase their reactivity and possible biochemical activity. Nanoparticles act by disrupting multiple cellular mechanisms including membrane permeability, ATP synthesis, and response to oxidative stress. Nanoparticles are synthesized using silver, gold, titanium oxide, silicon oxide, zinc oxide or biologicals which are referred as green nanoparticles. Scientists showed the nanoparticles of zinc oxide were antagonistic towards Meloidogyne incognita. Nanoparticles are produced by physical, biological and chemical processes. Use of gold nano-particles can reduced 100% infection of *M. incognita* reported use of CuFe NPs exhibited the highest nematocidal activity, causing 100% paralysis to *Meloidogyne* species. Nanoparticles are advantageous due to their higher efficiency and longer shelf life. However, their environmental impact assessment needs to be conducted before commercialization.

Introduction:

Nematode pests pose a significant threat to crop production worldwide, leading to substantial economic losses and food security concerns. These microscopic worms, known as plant parasitic nematodes (PPNs), infect the roots of various crops, interfering with nutrient uptake and causing stunted growth, wilting, and yield reduction. The impact of PPNs on agriculture is staggering, with an estimated annual global loss of US $173 billion (Elling, 2013). In India alone, these nematodes cause an average crop loss of 21.3% annually (Kumar *et al.*, 2020).

Overview of nematode pests and their impact on crop production

To set the stage for understanding the role of nanotechnology in nematode management, it is crucial to provide an overview of the different types of nematode pests and their impact on crop production. This section will introduce the major genera and species of PPNs that commonly infest agricultural crops worldwide. It will highlight the diverse symptoms exhibited

by infected plants and the subsequent yield losses experienced across various agricultural regions. The economic implications of nematode damage will be emphasized to underscore the urgency of effective management strategies.

Challenges associated with traditional nematode management approaches

Conventional methods used to manage nematode pests have limitations that hinder their effectiveness. This section will address the challenges faced by traditional nematode management approaches, such as chemical pesticides, cultural practices, and biological control methods. Despite their widespread use, chemical pesticides pose concerns regarding environmental contamination, detrimental effects on non-target organisms, and the development of pesticide resistance in nematodes. Cultural practices, such as crop rotation and sanitation, provide only partial control and are not always feasible for intensive commercial agriculture. Biological control agents, including nematophagous fungi and bacteria, have limited efficacy or inconsistent results.

The need for innovative and sustainable solutions is evident, prompting the exploration of nanotechnology as a potential game-changer in nematode management. Nanotechnology offers novel approaches and tools that can overcome the limitations of traditional methods, providing more efficient and environmentally friendly strategies for controlling nematode pests.

Nanotechnology: An overview

Definition and principles of nanotechnology

Nanotechnology involves the manipulation and control of matter at the nanoscale, typically ranging from 1 to 100 nanometers (nm) or 1.0 × 10^-9 meters. At this scale, materials exhibit unique properties and behaviors that differ from their bulk counterparts. The principles of nanotechnology revolve around the ability to engineer and harness these unique characteristics for various applications.

The two main principles of nanotechnology are:

a) Size-dependent properties: Materials at the nanoscale exhibit size-dependent properties due to their increased surface-to-volume ratio. This property leads to enhanced reactivity, increased surface energy, and altered physical, chemical, optical, and electrical characteristics. Size-dependent properties make nanoparticles highly versatile and advantageous for numerous applications.

b) Quantum effects: At the nanoscale, quantum effects become more pronounced, influencing the behavior and properties of materials. Quantum confinement, quantum size effects, and quantum dots are examples of phenomena observed in nanomaterials. These effects can be utilized to manipulate and control the properties of nanoparticles.

Properties and advantages of nanoparticles

Nanoparticles, the building blocks of nanotechnology, possess distinct properties that make them highly suitable for various applications, including nematode pest management. This section will highlight the key properties and advantages of nanoparticles, including:

a) High surface area-to-volume ratio: Nanoparticles have a significantly higher surface area relative to their volume. This property allows for increased interactions with target organisms, facilitating more efficient and targeted actions.

b) Enhanced reactivity: The high surface area of nanoparticles amplifies their reactivity, enabling them to interact with biological systems and disrupt cellular mechanisms effectively. This property is particularly advantageous in the context of nematode management.

c) Tailorable properties: Nanoparticles' properties can be tailored by modifying their size, shape, composition, and surface functionalization. This tunability provides flexibility in designing nanoparticles with specific characteristics optimized for nematode control.

d) Versatility: Nanoparticles can be synthesized from a wide range of materials, including metals (e.g., silver, gold), metal oxides (e.g., titanium oxide, zinc oxide), and even biologically derived materials. This versatility allows for the selection of nanoparticles that exhibit desired properties and compatibility with nematode management strategies.

Synthesis methods for nanoparticles

This section will discuss various synthesis methods employed to produce nanoparticles for nematode management. The synthesis of nanoparticles can be achieved through physical, chemical, or biological processes. Common synthesis methods include:

a) Physical methods: Physical methods involve techniques such as milling, vapor deposition, and lithography. These methods enable the production of nanoparticles with precise control over size, shape, and composition.

b) Chemical methods: Chemical methods utilize chemical reactions to produce nanoparticles. Examples include reduction, precipitation, sol-gel, and hydrothermal methods. These techniques offer versatility in synthesizing nanoparticles from different materials.

c) Biological methods: Biological methods, also known as green synthesis, employ living organisms or their extracts to produce nanoparticles. This eco-friendly approach utilizes biological entities such as plants, bacteria, fungi, or enzymes to reduce metal ions and fabricate nanoparticles.

The choice of synthesis method depends on the specific requirements of the nanoparticles and their intended application in nematode pest management. Each method has its advantages and limitations, and researchers continually explore novel approaches to enhance the efficiency and scalability of nanoparticle synthesis.

Nanoparticles for nematode management

Selection of nanoparticle materials for nematode control

The selection of nanoparticle materials plays a crucial role in designing effective strategies for nematode control. This section will discuss the factors considered when choosing nanoparticle materials for nematode management. Key considerations include:

a) Toxicity to nematodes: Nanoparticles should possess inherent toxicity or nematocidal properties to effectively target and control nematode populations. The toxicity can be attributed to specific physicochemical properties of the nanoparticles, such as size, surface charge, and composition.

b) Compatibility with the target crops: Nanoparticle materials should be compatible with the target crops to avoid any negative impact on plant health. It is essential to select nanoparticles that are safe for plants while exhibiting potent nematode control properties.

c) Environmental safety: The environmental impact of nanoparticles should be evaluated to ensure their safe application. Nanoparticles with low persistence and minimal adverse effects on non-target organisms and ecosystems are preferred.

d) Stability and delivery: Nanoparticles should maintain stability under field conditions and be capable of targeted delivery to nematode-infested areas. The choice of nanoparticle materials influences their stability and the development of appropriate delivery systems.

Efficacy of Zinc oxide nanoparticles against *Meloidogyne incognita*

Zinc oxide nanoparticles (ZnO NPs) have demonstrated significant nematocidal activity against *Meloidogyne incognita*, one of the major nematode species causing crop damage. This section will delve into the efficacy of ZnO NPs in nematode control, focusing on their effects on *M. incognita*. Key findings and research studies demonstrating the nematocidal activity of ZnO NPs will be discussed. This includes studies that highlight the disruption of nematode cellular mechanisms, such as membrane permeability, ATP synthesis, and oxidative stress response, by ZnO NPs. Furthermore, the potential mechanisms of action through which ZnO NPs exert their nematocidal effects will be explored. These mechanisms may involve direct interactions with nematode tissues, interference with nematode movement and feeding, or modulation of nematode gene expression.

Other types of nanoparticles and their nematocidal activities

In addition to zinc oxide nanoparticles, various other types of nanoparticles have shown nematocidal activities against plant parasitic nematodes. This section will discuss some examples of nanoparticles derived from different materials and their nematocidal activities. The focus will be on their effectiveness against nematode species, including *Meloidogyne* spp.

Examples of nanoparticles that have exhibited nematocidal activities include silver nanoparticles, gold nanoparticles, titanium oxide nanoparticles, and copper/iron nanoparticles. The section will highlight relevant studies showcasing the efficacy of these nanoparticles in controlling nematode populations. The specific mechanisms of action employed by these nanoparticles against nematodes will be explored, along with their advantages and limitations.

The discussion will emphasize the potential of diverse nanoparticle materials for nematode management and the need for further research to explore their effectiveness, optimize application methods, and ensure environmental safety.

Mechanisms of action

Disruption of cellular mechanisms by nanoparticles

Nanoparticles exhibit their nematocidal activity by disrupting various cellular mechanisms within nematodes. This section will delve into the specific cellular processes that are targeted by nanoparticles, leading to nematode control.

a) Membrane disruption: Nanoparticles can interact with nematode cell membranes, leading to their disruption or destabilization. This interference with membrane integrity can result in

impaired cellular functions and ultimately lead to nematode mortality.

b) Enzyme inhibition: Nanoparticles may interfere with specific enzymes involved in essential biochemical processes within nematodes. By inhibiting these enzymes, nanoparticles disrupt vital metabolic pathways, causing dysfunction and eventual nematode death.

c) DNA damage: Nanoparticles can induce DNA damage within nematode cells. This can lead to genetic abnormalities, impairments in replication and transcription, and overall disruption of cellular processes necessary for nematode survival.

d) Reactive oxygen species (ROS) generation: Nanoparticles can trigger the generation of reactive oxygen species within nematodes. ROS, such as superoxide radicals and hydrogen peroxide, cause oxidative stress and damage biomolecules like proteins and lipids, leading to nematode mortality.

Impact on membrane permeability, ATP synthesis, and oxidative stress response

Nanoparticles have been found to affect crucial cellular processes in nematodes, including membrane permeability, ATP synthesis, and the oxidative stress response. This section will provide an overview of how nanoparticles influence these mechanisms and contribute to nematode control.

a) Membrane permeability: Nanoparticles can disrupt the integrity of nematode cell membranes, resulting in increased permeability. This disruption compromises the selective passage of ions and molecules, disrupts osmoregulation, and disturbs cellular homeostasis. Ultimately, the loss of membrane integrity can lead to the death of nematodes.

b) ATP synthesis: Nanoparticles can interfere with ATP synthesis, which is vital for energy production and cellular processes in nematodes. By targeting enzymes or disrupting electron transport chains involved in ATP synthesis, nanoparticles can inhibit ATP production, leading to energy depletion and subsequent nematode mortality.

c) Oxidative stress response: Nanoparticles can induce oxidative stress within nematodes by promoting the generation of reactive oxygen species (ROS) beyond the nematode's antioxidant defense capacity. Excessive ROS production overwhelms the nematode's ability to neutralize them, resulting in oxidative damage to cellular components and ultimately leading to nematode death.

The disruption of these cellular mechanisms by nanoparticles underscores their potential as effective tools for nematode management. However, further research is needed to elucidate the precise mechanisms of action for different nanoparticle materials and their interactions with specific nematode species.

Advantages and applications

Enhanced efficiency of nanoparticles in nematode management

Nanoparticles offer several advantages over traditional nematode management approaches, leading to enhanced efficiency in controlling nematode pests. This section will highlight the specific advantages of nanoparticles in nematode management.

a) Increased efficacy: The unique properties of nanoparticles, such as their high surface area-to-volume ratio and enhanced reactivity, enable them to exhibit higher efficacy in targeting and

controlling nematodes. This increased efficacy can result in more effective pest management and reduced crop damage.

b) Targeted action: Nanoparticles can be designed and functionalized to specifically target nematodes while minimizing effects on non-target organisms. This targeted action allows for selective nematode control, reducing the risk of detrimental impacts on beneficial organisms.

c) Multiple modes of action: Nanoparticles can disrupt nematode cellular mechanisms through various modes of action simultaneously. For example, they can disrupt membranes, inhibit enzymes, and induce oxidative stress, providing a multi-faceted approach to nematode management.

Shelf-life extension and sustained release formulations

Nanoparticles can contribute to the development of shelf-life extension and sustained release formulations for nematode management products. This section will discuss the potential of nanoparticles in improving the longevity and controlled release of active ingredients.

a) Improved stability: Nanoparticles can enhance the stability of active ingredients, protecting them from degradation and ensuring their effectiveness over an extended period. This increased stability can prolong the shelf life of nematode management products.

b) Controlled release: Nanoparticles can be formulated to release their active ingredients in a controlled manner. This controlled release enables a sustained presence of the active compounds, ensuring long-term efficacy against nematodes. Controlled release formulations can also minimize the frequency of application, reducing costs and labor requirements.

c) Protection against environmental factors: Nanoparticles can provide a protective barrier around the active ingredients, shielding them from environmental factors such as UV radiation, temperature variations, and microbial degradation. This protection helps maintain the potency of the active compounds and extends their effectiveness in nematode control.

Potential for integrated pest management strategies

Nanoparticles hold promise for integration into broader pest management strategies, such as integrated pest management (IPM). This section will explore the potential applications of nanoparticles in IPM approaches for nematode control.

a) Reduced reliance on chemical pesticides: Nanoparticles offer an alternative to chemical pesticides, reducing the dependence on conventional chemical-based approaches. By incorporating nanoparticles into IPM strategies, the overall pesticide load can be minimized while achieving effective nematode control.

b) Synergistic effects: Nanoparticles can be combined with other nematode management approaches, such as cultural practices, biological control agents, and resistant crop varieties, to create synergistic effects. The integration of nanoparticles with these approaches can enhance the overall efficacy of nematode management strategies.

c) Environmental sustainability: Nanoparticles, when used judiciously, can contribute to environmentally sustainable pest management practices. Their targeted and controlled release capabilities, reduced pesticide use, and potential compatibility with biological control agents align with the principles of sustainable agriculture.

The application of nanoparticles in integrated pest management strategies offers the potential for more effective, sustainable, and environmentally friendly nematode control practices. However, further research is needed to optimize their formulation, application methods, and assess their long-term impacts on non-target organisms and ecosystems.

Environmental impact assessment

Importance of assessing environmental implications

Assessing the environmental impact of nanotechnology-based nematode management strategies is crucial to ensure their safe and sustainable implementation. This section highlights the significance of conducting comprehensive environmental impact assessments.

a) Ecological risks: Evaluating the potential impacts of nanoparticles on non-target organisms, such as beneficial insects, soil microorganisms, and aquatic organisms, is essential. It helps determine the potential risks associated with the use of nanoparticles and guides the development of mitigation strategies to minimize adverse effects on the environment.

b) Soil and water contamination: Nanoparticles used in nematode management have the potential to accumulate in soil and water systems. Assessing their persistence, mobility, and potential for leaching or runoff is necessary to understand their fate in the environment and potential contamination risks.

c) Long-term effects: Long-term studies are required to assess the cumulative effects of nanoparticles on ecosystems. This includes monitoring changes in soil health, biodiversity, and ecological processes over time to identify any potential long-term consequences of nanoparticle application.

Considerations for commercialization and regulatory aspects

Before the commercialization of nanotechnology-based nematode management products, regulatory aspects and risk assessment must be carefully considered. This section discusses key considerations in this regard.

a) Safety assessments: Rigorous safety assessments should be conducted to evaluate the potential risks associated with nanoparticle-based products. This includes toxicity testing, evaluation of environmental fate and behavior, and consideration of potential impacts on human health.

b) Regulatory frameworks: Existing regulatory frameworks may need to be adapted or expanded to encompass nanotechnology-based products. This involves establishing guidelines and standards specific to nanoparticle-based nematode management products to ensure their safe and responsible use.

c) Labeling and information dissemination: Clear labeling and appropriate information dissemination are essential to educate users about the proper handling, application, and potential risks associated with nanoparticle-based products. This helps promote responsible use and enables informed decision-making.

Future directions and challenges

Emerging trends in nanotechnology for nematode management

The field of nanotechnology for nematode management is continuously evolving, and several emerging trends offer promising directions for future research and development. This section discusses some of these trends:

a) Novel nanoparticle materials: Exploring new nanoparticle materials with enhanced nematocidal properties and reduced environmental impacts is a promising avenue. Research on innovative materials and their specific modes of action can contribute to the development of highly efficient and environmentally friendly nematode management solutions.

b) Nanoparticle delivery systems: Further advancements in nanoparticle delivery systems can improve targeted delivery and controlled release of active ingredients. The development of smart delivery systems that respond to environmental cues or specific nematode behaviors can enhance the overall effectiveness of nanoparticle-based nematode management.

c) Nanosensors for monitoring and detection: Nanosensors can provide real-time monitoring and detection of nematode populations in the field. The integration of nanotechnology with sensor technologies can enable early pest detection, allowing for timely and targeted interventions.

Addressing challenges and limitations

Several challenges and limitations need to be addressed to fully exploit the potential of nanotechnology for nematode management. This section highlights some key areas for improvement:

a) Standardization and validation: Standardizing nanoparticle synthesis methods, characterization techniques, and efficacy evaluation protocols is crucial for reliable and reproducible results. Consistent standards and validation procedures facilitate comparisons between studies and enable the development of robust nanotechnology-based nematode management strategies.

b) Cost-effectiveness: The cost of nanoparticle synthesis, formulation, and application can pose challenges for widespread adoption. Research should focus on developing cost-effective production methods and optimizing formulation strategies to ensure affordability and practicality for farmers.

c) Long-term environmental effects: Long-term monitoring and assessment of the environmental impacts of nanoparticle-based nematode management are necessary. Understanding the potential ecological consequences and implementing proactive measures to mitigate any adverse effects are essential for sustainable implementation.

Promising research areas and potential applications

Despite the challenges, nanotechnology holds great potential for various applications in nematode management. This section highlights promising research areas and potential applications:

a) Nanoparticles in seed treatments: Developing nanoparticle-based seed treatments can protect seedlings from nematode damage during germination and early growth stages. This

approach offers a preventive measure against nematode infestation and reduces the reliance on post-emergent control methods.

b) Nanoparticles in soil amendments: Incorporating nanoparticles into soil amendments, such as organic matter or biochar, can enhance their nematode control properties and improve their effectiveness in managing nematode populations in the soil.

c) Nanotechnology in nematode diagnostics: Nanotechnology-based tools, such as biosensors or nanoscale imaging techniques, can facilitate rapid and accurate detection and identification of nematode species. This enables targeted and precise management strategies based on specific nematode characteristics.

Conclusion:

Nanotechnology holds significant promise in the management of nematode pests, offering enhanced efficacy, controlled release formulations, and integration into sustainable pest management strategies. The unique properties of nanoparticles, their targeted action, and multi-faceted modes of action provide opportunities for more efficient and environmentally friendly nematode control. However, comprehensive environmental impact assessments, consideration of regulatory aspects, and addressing challenges and limitations are crucial for the safe and responsible commercialization of nanoparticle-based nematode management products. Continued research in emerging trends, such as novel nanoparticle materials, advanced delivery systems, and nanosensors, will further advance the field and contribute to effective and sustainable nematode management practices.

References:

1. Ch, G., Ntalli, N., Menkissoglu-Spiroudi, U. and Dendrinou-Samara, C. (2019). Essential Metal-Based Nanoparticles (Copper/Iron NPs) as Potent Nematicidal Agents against *Meloidogyne* spp. *Journal of Nanotechnology Research*, ***1***(2): 44-58.
2. Elling, A. A. (2013). Major emerging problems with minor *Meloidogyne* species. *Phytopathology*, ***103***(11): 1092-1102.
3. Kumar, V., Khan, M. R., and Walia, R. K. (2020). Crop loss estimations due to plant-parasitic nematodes in major crops in India. *National Academy Science Letters, **43***, 409-412.
4. Khan, A., Ali Khan, A., Jameel, M., Farhan Khan, M., Khan, M., Khan, A. and Alam, M. (2023). Grass-Shaped Zinc Oxide Nanoparticles Synthesized by the Sol-Gel Process and Their Antagonistic Properties towards the Biotrophic Parasite, *Meloidogyne incognita*. *Bioinorganic Chemistry and Applications*, 2023.
5. Thakur, R. K., Dhirta, B. andShirkot, P. (2018). Studies on effect of gold nanoparticles on *Meloidogyne incognita* and tomato plants growth and development. *BioRxiv*, 428144.
6. Worrall, E. A., Hamid, A., Mody, K. T., Mitter, N. and Pappu, H. R. (2018). Nanotechnology for plant disease management. *Agronomy*, 8(12), 285

PHYTOTHERAPEUTIC PROPERTIES OF HONEY - A NARRATIVE REVIEW

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Abstract:

Honey is a sweetening compound that can be stored and used exactly as produced in nature. It is a sweet, viscous substance produced by the different species of honey bees. Researchers and medical professionals have rediscovered some therapeutic uses of honey, especially where antimicrobial agents have failed to cure infections. There are many reports that show the antimicrobial activity of honey against pathogenic microorganisms. The antibacterial activity of honey is attributed to its osmolarity, H_2O_2 content, low pH, phenolic acids levels, and flavonoids. Phytochemical factors make honey active against pathogenic bacteria and provide either bacteriostatic or bactericidal efficacy. Different studies have evaluated the combination of honey with other bee derived products, such as propolis and royal jelly. The current chapter discusses antimicrobial activities of different types of honey as explored by various researchers.

Introduction:

Honey is a wonderful natural product that is formed from the nectar of flowers by honey bees which are build a honey hive. Honey is a sweetening compound that can be stored and used exactly as produced in nature. It is a sweet, viscous substance produced by the different species of honey bees. The honey combs are constructed by the bees from wax they secrete, the production of which requires about 8–10 times its weight in honey. The honey produced from predominantly single species of flowers is a monofloral honey and a honey that is composed of various pollens from different flower species is called a multifloral honey. Honey varies greatly in its colour, flavour, moisture content, sugar composition and its phytochemical properties.

From time immemorial, honey has been used by people around the world for its therapeutic benefits (1). The ancient Greeks promoted the use of honey for various ailments like acute fevers, baldness, eye disease, cough, sore throat, for topical antisepsis and in preventing and treating scars (2). Honey was used by ancient Egyptians, Greeks, Indians, and Chinese to treat wounds, infections, and various diseases. According to the Indian science of Ayurveda, honey is effective for treatment of skin diseases, pains, anemia, lung imbalances and cardiac related pains (3). The by-products of honey like bee pollens, royal jelly and bee venom have also been tested for their antimicrobial capacities.

Currently, researchers are shifting their attention to medicines which have natural origins, and they believe that some natural products may be as effective as newer synthetic drugs. Researchers and medical professionals have rediscovered some therapeutic uses of honey,

especially where antimicrobial agents have failed to cure infections (4). There are many reports that show the antimicrobial activity of honey against pathogenic microorganisms. Apitherapy is defined as a special discipline that studies all therapeutic products derived from honey bees (5). Presently, there are many types of 4 honey available which claim to have antibacterial properties. One of the most famous is Manuka honey, which is derived from Leptospermum scoparium trees found in New Zealand and eastern Australia.

Composition of honey:

Honey is a complex mixture of sugars and carbohydrates. It is primarily composed of fructose and glucose and the other sugars are maltose, sucrose, and other complex carbohydrates. Composition and properties like aroma, colour, and flavour and antioxidant nature of honey depends on the type of flowers, honey bee species, geographic locations, climate and flowering season and storage. Honey has low water content. When the moisture content increases it causes fungal and yeast formation leading to loss of properties of honey. Honey is a complex food that contains 181 different substances such as phenols, flavonoids, kojic acid, 5-hydroxymethylfurfural (HMF), tetracycline, fatty acids, peroxides, ascorbic acid, amylase, terpenes, phenols, benzoic acid and benzyl alcohols etc., The flavonoids present in honey are categorized into three classes with similar structure: flavonols, flavones and flavanones. These are contributed significantly to honey colour, taste and flavour and also have been beneficial for health effects. Diastase numbers (DN), hydroxymethylfurfural (HMF), proline and sucrose are usually used as indicators of the ripeness and quality of honey. Investigations have shown that a wide range of trace elements are present in honey, including Al, Ba, Bi, Co, Cr, Mo, Ni, Pb, Sn, Ti, as well as minerals (Ca, Cu, Fe, K, Na, Mg, Mn, Zn). The main mineral element is potassium while copper presents in lowest amount. Vitamins such as thiamine (B1), riboflavin (B2), pyridoxine (B6), and ascorbic acid (C) have also been reported but their amount is very small in honey.

The antibacterial activity of honey is attributed to its osmolarity, H2O2 content, low pH, phenolic acids levels, and flavonoids. Phytochemical factors make honey active against pathogenic bacteria and provide either bacteriostatic or bactericidal efficacy (6, 7). The essential phytochemicals and their biochemical analysis are briefed in table 1.

As agricultural practices and seasons change, the value of areas for beekeeping or the quality, type, and amount of honey produced will be influenced. The antioxidant activity of honey is very much dependent on the botanical origin where the bee was reared. Manuka honey has had major importance due to its broad antibacterial efficacy. Manuka honey is derived from Leptosperm sp. originating in New Zealand. This unifloral honey has been used in the pharmaceutical industry for the treatment of a variety of diseases and has been reverted to medical-grade honey (8, 9).

Table 1: Phytochemistry of honey and common methodology

Sr. No.	Test	Method
1.	Determination of the Honey Colour	According to the methodology proposed by the Codex Alimentarius Commission, (14) the colour of honey is determined by reading the absorbance of the pure honey sample in a spectrophotometer at 560 nm against pure glycerine blank.
2.	Determination of Acidity	According to AOAC (1998), (15) the total acidity of honeys is obtained by titrating the honey sample for free acidity with a solution of NaOH 0.05 mol L−1 , until it reaches a pH of 8.5. For lactonic acidity, after the solution reaches a pH of 8.5, 10 mL of 0.05 mol L−1 NaOH is pipetted and, with 0.05 mol L−1 HCl, the back titration is performed until pH 8.3.
3.	Determination of Moisture	According to AOAC (1998), (15) the moisture is determined by refractometry, where the refractive index of honey at 20 ◦C, and for each degree above the temperature that the sample presented, 0.00023 was added. The corrected refractive index was converted to moisture percentage using a reference table
4.	Determination of Reducing Sugars	According to the CAC method (16) from the modification of the Lane and Eynon procedure, involving the reduction of the Fehling solution, modified by Soxhlet, during the titration at boiling point with a solution of bee honey sugar reducers, using methylene blue as an indicator. The apparent sucrose content was determined after inversion by acid hydrolysis.
5.	Apparent Sucrose	An amount of 50 mL of the honey solution obtained in the determination of reducing sugars is pipetted into a 100 mL volumetric flask and 25 mL of water is added. Heating is carried out at 65 ◦C in a water bath. The flask is removed from the bath and 10 mL of hydrochloric acid solution is added and the solution is allowed to cool naturally to room temperature, then neutralized with sodium hydroxide solution. P = sample mass in g V1 = number of mL of diluted sample solution spent in the titration C = number of g of invert sugar percent, obtained before inversion, reducing sugars.
6.	Total Polyphenols	According to the Folin–Ciocalteu method, 1 g sample of honey sample is put in a 10 mL volumetric flask, which is completed with water and filtered through with paper weight 80 g/m2 . An amount of 0.5 mL of this solution is then added with 2.5 mL Folin–Ciocalteu reagent (0.2 n), and mixed for 8 min followed by the addition of 2 mL of sodium carbonate (75 g L−1). Then the mixture solution is allowed to incubate at room temperature for 2 h and the absorbance is measured at 760 nm, while methanol is used as blank. The total phenolic content is expressed in mg equivalent of gallic acid per 100 g of honey

Types of honey and its antimicrobial efficacy:

There are two types of honey available based on the floral resources used by bees; monofloral honey and polyfloral honey. Monofloral honeys come from single plant species providing the source of nectar, while polyfloral honeys are derive from nectars of multiple plant species (10). Monofloral honeys have characteristic aromas, which usually indicate that they contain volatile compounds that originate from the sources of nectar. Some monofloral honeys also have stronger antibacterial properties. Some pathogens are more susceptible to monofloral honeys. Zafar showed that when a monofloral honey was applied to a wound, bacterial attachment to the tissue was blocked, thereby inhibiting the formation of biofilms at the wound site (11). He also showed that when honey was used with antibiotics like oxacillin, it synergistically increased the antibiotic's effect. A resistant pathogen such as MRSA (Methicillin resistant Staphylococcus aureus) became susceptible to the combination of honey and oxacillin. Research has also shown that honey is effective in veterinary medicine, and has cured diseases like mastitis, foot and mouth infections, gastrointestinal disorders, and otitis (12). Honey has also shown antifungal activity and has been effective in treating dermatophytosis such as onychomycosis, and athlete's foot. Additionally, honey has shown antiviral effects. It has been used topically to successfully treat herpes simplex lesions, rubella rash, and varicella zoster lesions. Honey has also shown some anti-mycobacterial properties. In one study, Zafar showed that the addition of 10% or 20% honey to the medium, inhibited the growth of clinical TB isolates, but growth was not inhibited in media containing lower concentrations. Some have suggested that including honey in one's diet would be beneficial in preventing mycobacterial infections (13).

Antimicrobial activity and mechanism action:

The antibacterial and antifungal properties of different types of honey against different microbes have been researched widely. Well diffusion and agar diffusion assays are the common laboratory methods to estimate the bactericidal and bacteriostatic properties. Bacteria including aerobes, anaerobes from gram positive and gram-negative categories show sensitivity to honey in in-vitro studies. The antibacterial activity of honey could be attributed to various factors like its high osmotic nature and naturally low pH (3.2-4.5) ability to produce hydrogen peroxide and the presence of phytochemical factors such as tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohol and benzoic acid (14 – 18). Various types of bacteria, involved in wound infections like Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Klebsiella spp., Streptococcus faecalis and Pseudomonas aeruginosa are susceptible to honey regardless of their resistance to antibiotics. Studies have been conducted from across the globe especially the African continent on honey sourced from diverse geographical locations (19).

Honey can act as a bacteriostatic agent which stops the reproduction of cells, without necessarily killing them or a bactericidal agent that kills the bacteria. Depending on the concentration of honey used to treat antibiotic-resistant pathogens, the result can be bacteriostatic or bactericidal (20).

Studies showed that honey could be bactericidal to certain bacteria even in their highly resistant biofilm state (21). V. Bansal *et al.*, showed that 3-7% pasteurized honey and 4-10% Manuka honey were bacteriostatic, whereas at concentrations of 5-9% and 7-14%, respectively, bactericidal activity was achieved. However, when a sugar solution which was similar in composition to that of honey (artificial honey) was used, it was bacteriostatic at 20-30% and was not bactericidal at all (22). Previous research has shown that Manuka honey has specific antimicrobial activity which is due to a non-peroxide mechanism known as Unique Manuka Factor (UMF) (20). The other recognized medicinal honey, besides Manuka honey, is Tualang honey, which is found in the Malaysian forests. This honey is also receiving attention from researchers and scientists because of its medicinal properties which are similar to those of Manuka honey.

Research on antimicrobial activity of organic honeys is a novel approach and only a few studies exist regarding bioactivity or quality. Estevinho et al in 2012 evaluated the quality of 75 organic Erica species sourced from the Tras-Os-Montes region in Portugal along with measurement of bioactivity and content of phenols and flavonoids. They found that TPC (total phenol content) and TFC (total flavonoid content) were relatively high and similar to previous studies with non-organic Erica sp. honeys (23).

Garedew *et al*. and Boorn *et al*. conducted separate research on Trigona species of honey bee and found that the minimal inhibition concentration (MIC) values of gram-positive bacteria were between 1 and 32% and of gram-negative bacteria was between 4 and 32% (24, 25).

Determination of the MICs of different stingless bee honeys reaffirmed the data obtained from the AWD assay, showing that Gram-positive strains are more sensitive than Gram-negative bacteria are. The gram-positive bacteria, Staphylococcus aureus also showed good MIC values in most of the studies. Different studies have evaluated the combination of honey with other bee derived products, such as propolis and royal jelly. In a couple of studies, positive interactions were observed between honey and bee derived products, but these can be considered as additive according to the fractional inhibitory concentration (FIC) index. Nishio et al conducted a study evaluating honey from two stingless bee species namely: *Scaptotrigona bipunctata* Lepeletier, 1836, and *S. postica* Latreille, 1807. Their results showed that the combination of the both the honeys involved a synergistic interaction, attaining an equal or even a better effect for a lower expense. The kinetic evaluation of the antibacterial effect of stingless bee honeys from both honeys showed a bactericidal effect. When both honeys are used in conjunction, the time required to eliminate all bacteria is shorter than when the honeys are applied separately, showing a great advantage of their combination (26). Similar results were observed by Temaru et al. who evaluated honey from three stingless bee honeys namely: Melipona beecheii, Trigona biroi and Scaptotrigona pectoralis.

Evaluation of the non-peroxide activity of honey by Kuncic et al showed that when treated with catalase, Slovenian honey had 20-fold increased MIC values against Gram-positive and Gram-negative bacteria (27). In study by Nishio *et al* (2016), when honey was treated with catalase, a 5-fold increase in the MIC values was documented. These data showed the

importance of hydrogen peroxide to the antibacterial activity of the honeys used in their work, yet there are also other components present in these honeys that may inhibit bacterial growth. Melipona compressipes manaosensis honey exhibits different antibacterial activity against Gram-positive and Gram-negative bacteria, depending on the season in which the honey is collected (wet or dry). The honey samples collected in the dry season were found to have a higher activity. Certain studies have analysed the physicochemical composition of honeys from stingless bee species (Scaptotrigona bipunctata Lepeletier and S. postica Latreille) and have showed difference in their composition based on pH values (4.17 and 3.4), free acidity (34.63 and 83.7 meq kg−1), and hydroxymethylfurfural (HMF) content (2.5 and 18.9mg kg−1) (28, 29).

Studies have also stated that the antibacterial activity varies according to the phytogeographic region, which yields different composition of honey. Recent studies have indicated the presence of other antimicrobial components such as methylglyoxal, antimicrobial peptide beedefensin-134, HMF 35 and phenolic compounds such as flavonoids (30, 31). Analysis of SEM micrographs enable us to observe morphological alterations caused by compounds placed in contact with the target bacteria. In studies which used SEM to observe alterations in bacterial morphology, a large number of cells formed septa, suggesting that effects on the cells make completion of the process of cell division unfeasible. Jenkins et al exposed MRSA to Manuka honey and observed similar results by transmission electron microscopy. These authors suggested that honey may act on an enzyme called “murein hydrolase”. The mechanism of action is triggered by interfering with the post-translational modification, preventing hydrolysis of cell wall components. This leads to a decrease in this enzyme’s activity and that causes failure in cell separation. Jenkins et al further stated that synergy of methylglyoxal with other components present in honey may also be responsible for honey’s anti-microbial activities (32).

Conclusion:

The World Health Organization has described alternative medicines as a cost-efficient way to achieve total health care coverage of the world’s population and has encouraged the rational use of plant-based alternative medicines by member states. Honey has been proven as an effective alternative for conventional medicines due to its proven anti-microbial efficacy. Further studies using honey-based interventions should be conducted to explore the patient compliance and rule out adverse effects for various systemic conditions. Indian honeys from diverse geographical locations have to be assessed for their anti-oxidant, anti-microbial and anti-fungal properties.

References:

1. Mandal S, Pal NK, Chowdhury IH, Deb Mandal M. (2009). Antibacterial activity of ciprofloxacin and trimethoprim, alone and in combination, against Vibrio cholerae O1 biotype El Tor serotype Ogawa isolates. Polish J Microbiol; 58:57–60. 39.
2. Zumla, A., and Lulat, A. (1989). Honey - a remedy rediscovered. Journal of the Royal Society of Medicine, 82(7), 384–385.

3. Eteraf-Oskouei, T., and Najafi, M. (2013). Traditional and modern uses of natural honey in human diseases: A review. Iranian Journal of Basic Medical Sciences, 16(6), 731–742.
4. Abuharfeil, N., Al-Oran, R., and Abo-Shehada, M. (1999). The effect of bee honey on the proliferative activity of human B- and T-lymphocytes and the activity of phagocytes. Food and Agricultural Immunology, 11(2), 169–177
5. Mandal, M. D., and Mandal, S. (2011). Honey: Its medicinal property and antibacterial activity. Asian Pacific Journal of Tropical Biomedicine, 1(2), 154–160.
6. Naureen et al. (2022) Antimicrobial and Antioxidant Activities of Honey. American Academic Scientific Research Journal for Engineering, Technology, and Sciences. 87(1):204-217.
7. Mavric, E. Wittmann, S. Barth, G. Henle, T. (2008) 'Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand. Mol. Nutr. Food Res. 52: 483–489.
8. Bucekova et al. (2019) ''Anti-bacterial Activity of Different Blossom Honeys'': New Findings. Molecules, 24: 1573.
9. El Sohaimy, S.A. Masry, S.H.D. Shehata, M.G. (2015) Physicochemical characteristics of honey from different origins. Ann. Agric. Sci. 60: 279–287.
10. Rossano et al (2012). What Are the Proteolytic Enzymes of Honey and What They Do Tell Us? A Fingerprint Analysis by 2-D Zymography of Unifloral Honeys. PLoS ONE, 7(11), 1–17.
11. Zafar, I. (2014). antimicrobial properties of honey. American Journal of Therapeutics, 21(4), 304–323.
12. Allen, K. L., and Molan, P. C. (1997). The sensitivity of mastitis-causing bacteria to the antibacterial activity of honey. New Zealand Journal of Agricultural Research, 40(4), 537–540
13. Mundo, M. A., Padilla-Zakour, O. I., and Worobo, R. W. (2004). Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. International Journal of Food Microbiology, 97(1), 1–8.
14. Codex Stan. Revised Codex Standard for Honey. Codex Stan 12-1981, Rev.1 1987, Rev. 2 2001. World Health Organization, Food and Agriculture Organization of United Nations; Roma, Italia: 2001.
15. Royo ET AL. (2022). Physicochemical Profile, Antioxidant and Antimicrobial Activities of Honeys Produced in Minas Gerais (Brazil). Antibiotics (Basel, Switzerland), 11(10), 1429. https://doi.org/10.3390/antibiotics11101429
16. Aljohar et al (2018). Physical and chemical screening of honey samples available in the Saudi market: An important aspect in the authentication process and quality assessment. Saudi pharmaceutical journal: SPJ: the official publication of the Saudi Pharmaceutical Society, 26(7), 932–942. https://doi.org/10.1016/j.jsps.2018.04.013
17. Kwakman P, Velde A, Boer L, Speijer D, Vandenbroucke-Grauls C, Zaat S. (2010). How honey kills bacteria. FASEB J., 24: 2576-2582. DOI:10.1096/fj.09-150789

18. Subrahmanyam M, Hemmady A, Pawar SG. (2001). Antibacterial activity of honey on bacteria isolated from wounds. Ann. Burns Fire Disasters, 14: 198-201
19. Teke and Ngienyikeh. (2016) Antibacterial activity of three Cameroonian honey types against some pathogenic species. Int. J. Biol. Chem. Sci. 10(6): 2477-2484.
20. Patton, T., Barrett, J., Brennan, J., and Moran, N. (2006). Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey. Journal of Microbiological Methods.
21. Wang, R., Starkey, M., Hazan, R., and Rahme, L. G. (2012). Honey's ability to counter bacterial infections arises from both bactericidal compounds and QS inhibition. Frontiers in Microbiology, 3(APR), 1–8.
22. Bansal, V., Medhi, B., and Pandhi, P. (2005). Honey--a remedy rediscovered and its therapeutic utility. Kathmandu University Medical Journal (KUMJ), 3(3), 305–309.
23. Obey. Jackie. (2022) Antimicrobial activity of commercial organic honeys against clinical isolates of human pathogenic bacteria. Org. Agr. 12:267–277.
24. Garedew, A., Schmolz, E. and Lamprecht, I. (2003) The antimicrobial activity of honey of the stingless bee Trigona spp. J Apicultural Sci. 47, 37–49.
25. Boorn, K. et al. (2010) Anti-microbial activity of honey from the stingless bee Trigona carbonaria determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology. J Appl Microbiol. 108, 1534–1543.
26. E. K. Nishio et al. (2016) Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata Lepeletier, 1836, and S. postica Latreille,1807;6:21641.
27. Kuncic, M. K., Jaklic, D., Lapanje, A. and Gunde-Cimerman, N. (2012) Antibacterial and antimycotic activities of Slovenian honeys. Br J Biomed Sci. 69, 154–158.
28. Souza, B. et al. (2006) Composition of stingless bee honey: Setting quality standards. Interciencia. 31, 867.
29. Borsato, D. M. (2013) Composição química, caracterização polínica e avaliação de atividades biológicas de méis produzidos por meliponíneos do Paraná (Brasil) Master's thesis, Universidade Federal do Paraná.
30. . Carina, L., Soledad, V. and Marina, B. (2014) Antibacterial activity of honey: A review of honey around the world. J Microb Antimicrob. 6, 51–56.
31. DeMera, J. H. and Angert, E. R. (2004) Comparison of the antimicrobial activity of honey produced by Tetragonisca angustula (Meliponinae) and Apis mellifera from different phytogeographic regions of Costa Rica. Apidologie. 35, 411–417.
32. Jenkins, R., Burton, N. and Cooper, R. (2011) Manuka honey inhibits cell division in methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother. 66, 2536–2542.

POLYHYDROXYALKANOATES AND THEIR IMPLICATIONS FOR SUSTAINABLE FUTURE

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Introduction:

The rising human civilization has recorded an increasing demand for the use of plastics. Synthetic biopolymers have become indispensible for day to day life owing to its broad spectrum use in every sectors including agriculture, medical, packaging, textile, automotive, marketplaces, households etc. (Phiri *et al.*, 2023). Recent decade has witnessed a relentless growth in plastic production exceeding 359 million tons by 2018 (Chia *et al.*, 2020). Most of these synthetic plastics are petroleum based polymers which pose a potential threat to the environment due to their non-biodegradable nature; and their production exhausts the non-renewable resources as well (Nagalakshmaiah *et al.*, 2019). Improper waste disposal inescapably introduces plastic polymers into the environment causing a sheer deterioration of natural resources (Hou *et al.*, 2021). Plastics can only be torn into pieces by physical, chemical, biological or mechanical processes and remain this way in the environment. The smaller plastic fragments with a size <5mm are referred to as "microplastics", which in recent time has created a global concern for its harmful impact on ecological niche (Zhang *et al.*, 2021). Hence, a plausible alternative for plastic is of great importance for a sustainable socio-economic future.

Microorganisms have always been proved to be the most attractive sources for a diverse kind of compounds. Polyhydroxyalkanoates or PHAs comprise a group of naturally occurring biodegradable polyesters, synthesized by a wide variety of microorganisms which include bacteria, algae and fungi (Singh Saharan *et al.*, 2014; Behera *et al.*, 2022). The term "bioplastic" is currently in use for PHAs which comply with the fact that they are derived from the renewable sources like vegetable oil, proteins, starch, food wastes etc. and can be successfully catabolized by microbes (Verlinden *et al.*, 2007). Due to their biodegradability and biocompatibility PHAs have drawn immense attention of researchers. Recent reports documented isolation and characterization of PHAs from yeasts, seaweed and plants (Behera *et al.*, 2022). The first microbial polyester was investigated by Lemoigne, who isolated the reserve polymer in *Bacillus megaterium* in 1926 and characterized it to be poly-(R)-(3-hydroxybutyrate) or P-(3HB). P-(3HB) was long thought to be the only type of polyester produced by microorganisms until polyesters containing 3-hydroxyalkanoate units longer than P-(3HB) were isolated from microorganisms by Wallen and Rohwedder (1974) in sewage sludge. According to a study, activated sludge showed the presence of polyesters like 3-hydroxyvalerate (3HV) and 3-

hydroxyhexanoate (3HHx) in addition to P-(3HB) (Shen *et al.,* 2015). A wide variety of aerobic and anaerobic heterotrophic bacteria including *Azotobacter beijerinckii*, *Clostridium butylicum*, *Alcaligenes eutrophus*, *Caryophanon latum*, *Pseudomonas* sp. have been shown to synthesize numerous polyesters of varying chain length (Anjum *et al.,* 2016). The properties of PHAs exclusively depend on the substrate, chemical composition and synthesis process of individual polymer type. Microorganisms synthesize PHAs in form of intracellular inclusion bodies for using them as carbon or energy reserve under nutrient limiting condition (Muigano *et al.,* 2023). Commercial production of PHAs employs fermentation technology using typical microbial strains and nutrient combination which in turn trigger the cells for inducing the PHA biosynthesis pathway. The fermentation is followed by precipitation, recovery and purification of the respective biopolymer from the media (Anjum *et al.,* 2016). There are several reports for *in vitro* production and recovery of different types of biopolymers from various microorganisms; however, large scale production of PHAs is still remaining a challenge due to various issues. Hence, comprehensive study on strain selection, media formulation, recovery and purification process of PHAs should be rigorously practiced for an eco-friendly future. This chapter summarizes the chemical nature, properties, fermentative production and applications of PHAs in order to focus attention on exploiting microbes for replacement of synthetic polymers.

Chemical nature of PHAs

PHAs are aliphatic polyesters which mainly are composed of C, H and O. These polyesters are formed by the action of microbial enzymes during their metabolic activity while utilizing carbohydrates, lipids and other renewable resources. The general chemical formula of PHA is represented in Figure 1. As documented in previous reports (Li *et al.,* 2016) PHAs are classified depending on the number of carbon atoms present in each monomeric unit and have been put into the following groups:

- Short chain length polyhydroxyalkanoates (SCL), consisting of 3-5 carbon atoms;
- Medium chain length polyhydroxyalkanoates (MCL), consisting of 6-14 carbon atoms and
- Long chain length polyhydroxyalkanoates (LCL) those which contain at least 15 carbon atoms.

Table 1: Names of some PHAs with their carbon chain length and category

Category	Carbon number	Name of the PHA
Short-chain-length (SCL)	C_3-C_5	Poly-3-hydroxybutyrate Poly-3-hydroxyvalerate Poly-3-hydroxybutyrate-co-3-hydroxyvalerate
Medium-chain-length (MCL)	C_6-C_{14}	Poly-3-hydroxyoctanoate Poly-3-hydroxynonanoate
Long-chain-length (LCL)	C_{15}-C_{18}	Poly-3-hydroxypentadecanoate Poly-3-hydroxyhexadecanoate

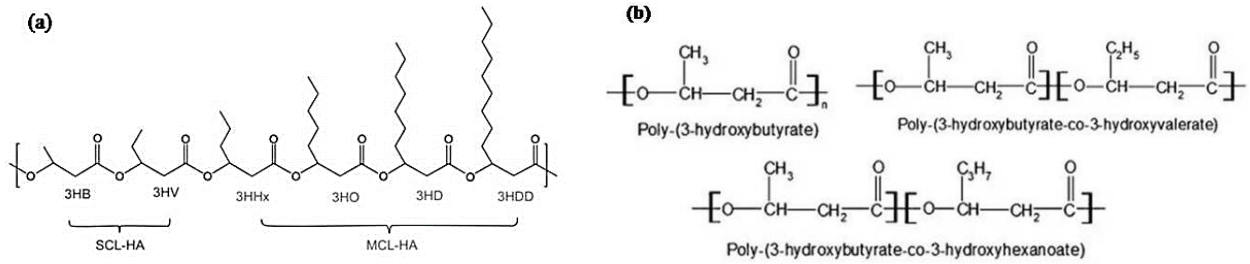

Figure 1: Structures of polyhydroxyalkanoates (PHAs); (a) General chemical formula of PHA, (b) Molecular structures of selected PHAs

Till date more than 150 types of microbial polyesters have been isolated and identified making them the largest group of natural polyesters (Sehgal and Gupta, 2020). Examples of some of the PHAs along with their respective category are presented in Table 1 and few of their structures are given in Figure 1.

Biosynthesis of PHA

PHA synthesis by living organisms is a natural course of their metabolism. Bacteria accumulate PHAs in their cytoplasm in form of insoluble inclusion granules when depletion in certain types of nutrients occurs (Shabina *et al.*, 2015). Microbial polyester synthesis can be attributed to the broad substrate specificity of the enzyme PHA synthase (PhaC) (Yasin and A-Mayaly, 2021). PhaC is the most important enzyme used in the biosynthesis of PHAs as it is responsible for the incorporation of various hydroxyacyl-CoAs into growing PHA chain. The operon *phbC-A-B*, identified in *A. eutrophus*, was the first evidence of the molecular mechanism for the synthesis of microbial polyesters (Tsuge *et al.*, 2005). Three important enzymes encoded by the structural genes present in this single operon play pivotal role in PHA biosynthesis. The three enzymes of this pathway are PHA synthase, encoded by *phbC* gene, 3-ketothiolase, encoded by *phbA* gene, and acetoacetyl-CoA reductase, encoded by *phbB* gene. PHB was the first polyester to be identified and extensively studied in bacteria. Bacteria utilize sugars and form acetyl CoA which undergoes three enzyme catalyzed reactions to produce PHB. The first step of PHB biosynthesis is characterized by the condensation of two molecules of acetyl CoA to form acetoacetyl CoA by the enzyme 3-ketothiolase.

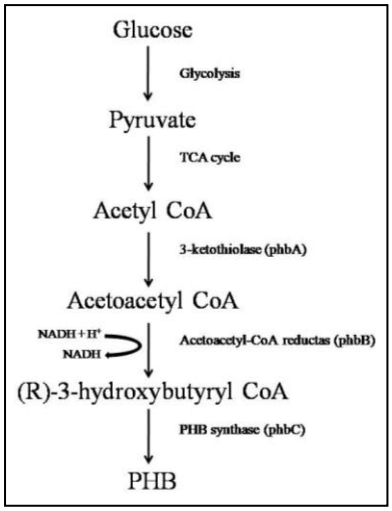

Figure 2: Biosynthesis of PHB

The next step is catalyzed by acetoacetyl-CoA reductase where acetoacetyl CoA is reduced to 3–hydroxybutyryl CoA with the investment of NADH. In the final step PHA synthase polymerizes 3–hydroxybutyryl CoA to PHB (Verlinden *et al.*, 2007). The biosynthesis steps are summarized in Figure 2.

Microbial production of polyhydroxyalkanoates

Microorganisms as PHA producers

Almost all types of microorganisms such as bacteria, fungi and microalgae have exhibited enormous potential for synthesising polyesters such as PHAs (Bernard, 2014). Till date, several PHA producers have been documented and these can be found under various ecological niches such as rhizosphere, agricultural, dairy, pulp and paper mill waste, sites contaminated with hydrocarbon, activated sludge and industrial effluents. Generally, nutrient limiting conditions with excess of carbon is the most suitable factor for PHA production (Chodak, 2008). Several important bacterial, fungal and microalgal sources are enlisted in Table 2 for the production of different types of PHA polyesters (Muneer *et al.*, 2020).

Table 2: Microorganisms producing PHAs

Bacteria	**Fungi**
Actinomycetes sp.	*Aspergillus fumigates*
Alcaligenes latus	*Arxula adeninivorans*
Aeromonal hydrophila	*Pichia pastoris*
Halobacterium	*Saccharomyces cerevisiae*
Azospirillum brasilense	*Yarrowia lipolytica*
Azatobacter vinelandii	*Wickerhamomyces anomalus*
Bacillus licheniformis	**Microalgae**
Burkholderia caryophylli	*Synechococcus subsalsus*
Methylobacterium sp. GW2	*Chlorella minutissima*
Chromobacterium Violaceum	*Synechocystis salina*
Pseudomonas aeruginosa 47T2	*Spirulina platensis*
Pseudomonas fluorescens	*Nostoc muscorum*
Rhodococcus ruber	*Aulosira fertilissima*
Staphylococcus sp.	
Streptomyces aureofaciens	

PHA production by Plant Growth Promoting Rhizobacteria (PGPR)

As different microbial communities interact intensely with each other in the soil-rhizosphere region, the recycling process of nutrients can take place in the environment in a coordinated manner. Many PGPR bacteria such as *Lysobacter gummosus, Burkholderia terricola, Azospirillum brasilense, Pseudomonas extremaustralis, Pseudomonas brassicacearum* and *Pseudomonas orientalis* have already been reported to accumulate PHA in soil (Gasser *et al.*, 2009). According to Prieto *et al.*, (1999), *Pseudomonas oleovorans* can accumulate PHA well enough in a media containing alkane as carbon source.

Photosynthetic bacteria as PHA producers

Screening of some cyanobacteria has already been reported for their ability to store PHAs. It has been found that production of PHB can also occur under phosphorus limiting conditions and in presence of excess of reducing equivalents (Philippis *et al.,* 1992). *Synechococcus* sp. MA19 (accumulated up to 55% of Cell Dry Weight), *Nostoc muscorum*n and *Spirulina platensis* produced PHB under phosphate limited conditions (Panda *et al.,* 2005). *Synechocystis sp.* PCC 6803 when subjected to nitrogen, phosphorus, and gas-exchange limiting conditions, PHB production was enhanced (Panda *et al.,* 2007).

Role of antibiotic producers in PHA synthesis

Studies suggest that PHBs serve as suppliers of acetoacetyl-Co-A and butyryl-Co-A for the production of antibiotic chloramphenicol in *Streptomyces venezuelae* and macrolide ascomycin FK520 in *Streptomyces hygroscopicus*, respectively. PHB being a primary metabolite serves as building blocks for the synthesis of metabolites (Singh Saharan *et al.,* 2014).

Hydrocarbon degraders as PHA producers

According to Dalal *et al.,* (2010), oil contaminated sites can be good reservoirs of PHA producing microorganisms such as *Burkholderia, Sphingobacterium, Brochothrix, Acinetobacter, Caulobacter, Ralstonia, Pseudomonas,* and *Yokenella*. Extracellular deposition of PHB by mutant of marine bacterium *Alcanivorax borkumensis* SK2 has been reported to grow on aliphatic hydrocarbon by Sabirova *et al.* (2006).

PHA production by halophiles

In 1970, an archaea named *Halobacterium marismortui* had been isolated from Dead Sea and analysed for their PHB accumulation. Extremely halophilic archaebacteria (Halobacteriaceae) produce PHB under nutrient-limited conditions and abundant carbon sources. *Haloferax mediterranei* produces 60 to 65% PHA of its cell dry weight (CDW) when grown in 25% (w/v) of salt concentration and in phosphate limiting conditions using glucose or sucrose as the best carbon source. *Halomonas boliviensis* LC1, when grown on starch hydrolysate as substrate and at 3–15% (w/v) of salt concentration, produces high amount of PHB up to 56% of Cell Dry Weight (Singh Saharan *et al.,* 2014).

PHA production by activated sludge

Industrial wastes containing high organic pollutants and low nutrient contents favor the production of PHAs. Wastes often require physical, chemical or mechanical treatment. Treatment can modify the structure of complex materials into simpler forms like volatile fatty acids (VFAs) and sugar moieties which are easy to uptake and can be stored by cells. Enrichment of activated sludge can influence the composition of VFAs, which in turn, influences the structure of the polymer. Tripathi *et al.* (2012) reported the production of PHA from *Pseudomonas aeruginosa* under submerged fermentation process utilizing sugar refinery waste (cane molasses) and about 62.44% production have been accounted. *Burkholderia cepacia* IPT 048 and *Bacillus sacchari* IPT 101 use hemicellulosic feedstock (sugarcane bagasse) after acid hydrolysis and boiling and it has been estimated that about 62% PHB can be produced by *Burkholderia sp.* and 53% by *Bacillus sp* (Silva *et al.,* 2004). Glycogen accumulating organisms

can utilize VFAs as the main constituents of sugarcane molasses to produce PHB in moderate to good quantity (Singh Saharan *et al.,* 2014).

Fermentative production of PHA

Microbial polyesters could be produced from leftover biomass used in various industries. Glucose, sucrose, starch, coconut oil, sugar beet and cane molasses, oleic acid, olive oil distillate are the best studied carbon sources, whereas some of the most common nitrogen supplements include $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3. For fermentative production of PHA, the culture of specific microorganism is inoculated in a suitable medium and fed appropriate nutrients so that it multiplies rapidly. The biosynthesis of PHA usually caused by certain deficiency conditions (e.g., lack of macro elements such as phosphorus, nitrogen, trace elements, or lack of oxygen) and with the excess supply of carbon sources. PHAs are deposited in form of granules whose nature varies with the type of strain and cultivation conditions. In this way both homo and co-polyesters can be generated (Ibrahim and Steinbüchel, 2009).

Recovery and purification of PHA from microbial cells

Solvent extraction

Two main steps are involved in solvent extraction, first is the modification of cell membrane permeability thus allowing release and solubilisation of PHA. This is then followed by non-solvent precipitation. Extraction of PHA with solvents such as chlorinated hydrocarbons, i.e. chloroform, 1,2-dichloroethane or some cyclic carbonates like ethylene carbonate and 1,2-propylene carbonate is common. Jiang *et al.,* (2006) studied that lower chain ketone such as acetone was found to be the most prominent solvent for the extraction of MCL-PHA. Methanol and ethanol induced precipitation of PHA is also able to remove bacterial endotoxin and causes negligible degradation to the polymers and thus PHA with high grade purity can be obtained.

Chemical and enzymatic digestion methods

Well established approaches for different digestion methods have been employed as an alternative to solvent extraction methods and can be classified into either chemical digestion or enzymatic digestion. A range of surfactants has also been evaluated such as sodium dodecyl sulfate (SDS), Triton X-100, palmitoyl carnitine, betaine and among them, SDS showed good performance. Surfactant-chelate digestion (Triton X-100 and ethylenediaminetetraacetic acid [EDTA]) could isolate PHA with 90% purity from enzymatically hydrolyzed cells of *Sinorhizobium meliloti* (Lakshman *et al.,* 2006).

Enzymatic digestion involves a rather complex procedure. Lakshman *et al.,* (2006), introduced *Microbispora sp*., a protease producing culture, to the fermented broth of *S. meliloti* and incubated for 72 h. The *S. meliloti* cells were then hydrolyzed by the protease and released PHA granules with other intracellular components. Application of a simple filtration process resulted in removal of lysed cells and 94% pure PHA. Extraction of pure enzyme is an expensive process, so nowadays a better alternative is whole cell enzyme.

Mechanical disruption

This process involves bead milling and high pressure homogenization. Bead milling employs the mechanism of shearing action and energy transfer from beads to cells in the contact zones. The key parameters that affect the disruption process are the bead loading and bead

diameter. The extent of cell disruption also depends on numerous parameters such as residence time distribution (RTD), shear forces, type of microorganisms, cell concentration, feed rate of the suspension, agitator speed, geometry of the grinding chamber and design of the stirrer. Disruption of cell suspension can also occur under high pressure through an adjustable orifice discharge valve. Process parameters such as operating pressure, number of passes, suspension temperature and homogenizer valve design must be carefully scrutinized for efficient disruption. Poly-3-Hydroxybutyrate with 95% purity and 98% yield was recovered from 5% (w/v) SDS pretreated *Methylobacterium* sp V49 cells subjected to homogenization at an operating pressure 400 $kgcm^{-2}$ after two cycles and this data was documented by Ghatnekar *et al.* (2002).

Flotation

Ibrahim and Steinbüchel, 2009 has investigated the recovery of PHB from a recently isolated bacterium, *Zobellella denitrificans* MW1. Simple extraction with various organic solvents followed by self-flotation of cell debris was also tested. The cells were mixed with chloroform at 30°C for 72 h and later subjected to self-flotation of cell debris overnight at room temperature. This method efficiently recovered 85% (w/w) of PHB with purity of 98%.

Supercritical fluid extraction

Supercritical-carbon dioxide ($SC–CO_2$) is the most predominantly used SCF due to its low toxicity and reactivity, moderate critical temperature and pressure (31°C and 73 atm), availability, low cost, and non-flammability. Using this method, approximately 89% PHB recovery was recorded from *Cupriavidus* sp (Hejazi *et al.,* 2003).

Gamma irradiation

Radiation induced cell disruption is independent of any chemicals resulting in relatively contamination free process. This process also promotes optimal disruption of cells at low dosage of irradiation which enable easier recovery of PHA. From irradiated wet cells of *Bacillus flexus*, PHA recovery of 54% (based on biomass dry weight) was attained within a short period. Efficacy of each of these techniques vary largely depending on the cellular constituents, types of PHA produced, solvent usage, working conditions and so on, thereby requiring extensive trial to prove their efficiency.

Applications of PHAs

PHAs have gained importance due to its non-toxic nature and ability of biodegradation compared to conventional plastics. Hence, microbial polyesters find immense applications in various fields like agriculture, packaging, molding products, medical devices, paper coating and many more. Their potential for replacing synthetic polymers has been extensively exploited by scientists.

Biomedical application

Medical industry employs various devices which are made up of plastics. With discovery of PHAs people started thinking about replacing the key components of those devices with microbial polyesters. PHAs have replaced conventional plastics for use in many ways including tissue culture implants, sutures, surgical implants, wound dressings, heart valves and pins etc. (Behera *et al.,* 2022). Many genera of bacteria e.g., *Pseudomonas* can depolymerize PHAs to monomeric compounds which show potential antibacterial property. Nowadays, scaffolds used in

tissue engineering are also made with PHAs as they provide better mechanical strength. Biomaterial mediated infection is of frequent occurrence in patients; therefore biodegradability of PHAs make them a promising alternative for plastic implants (Ray and Kalia, 2017).

Pharmacological application

Recent understanding of drug delivery systems demand controlled targeted approach for delivery of specific drugs. PHAs have been proved to be the biomaterial having requisite physical properties to be used as raw material for tablets and nano-particle mediated drug delivery system.

PHAs already have displayed high degree of biocompatibity and efficacy in drug delivery, protein purification and immobilizing agents for clinical purpose (Zhang *et al.,* 2018).

Food industry

In a report by Ragaert *et al.* (2019), bacterial polyesters have been shown to have considerable permeability for gases and water vapors which allow them to be used as packaging material in food industries.

Agriculture

For encapsulation of fertilizers PHAs are used in recent time. Other than this, agricultural industries use plastic products for various purposes all of which can be replaced by these biodegradable polyesters (Amelia *et al.*, 2019).

Environmental

The incidence of deadly diseases has increased sharply which on greater extent is related to environmental pollution by plastics. Current researches are therefore steered on developing essential commodities with biodegradable plastics. Bags, bottles, disposable materials, materials of personal hygiene, all can be made using bioplastics with careful management of production process. Different types of monomers and oligomers are also recovered for the production of larger polymers with the help of biodegradable polyesters (Wang and Chen, 2017).

Conclusion:

PHAs are naturally occurring polymers synthesized by various microorganisms and plants. The remarkable properties of PHAs have made them a candidate of choice for establishing an eco-friendly environment and thereby diminishing the detrimental effect of plastic pollution. Various approaches have been adopted till date for modifying these polyesters to increase their thermodurability and other features. A great variety of organisms showed accumulation of PHAs with pretty good yield. By proper manipulation of nutritional and growth conditions microorganisms can be used for extraction of microbial polyesters. However, certain challenges like high production and extraction cost, limit their use on commercial scale. In order to overcome these difficulties; careful selection of fermentation medium, optimization of extraction condition, strain selection, better understanding of PHA synthesis kinetics have to be investigated and standardized.

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References:

1. Amelia TSM., Govindasamy S., Tamothran AM., Vigneswari S., Bhubalan K. (2019): Applications of PHA in Agriculture, In: Kalia, V. (eds) Biotechnological Applications of Polyhydroxyalkanoates, Springer. https://doi.org/10.1007/978-981-13-3759-8_13.
2. Anjum A., Zuber M., Zia KM., Noreen A., Anjum MN., Tabasum S. (2016): Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of recent developments, International Journal of Biological Macromolecules, 89:161–174.
3. Behera S., Priyadarshanee M., Vandana, Das S. (2022): Polyhydroxyalkanoates, the bioplastics of microbial origin: Properties, biochemical synthesis, and their applications, Chemosphere, 294:133723.
4. Bernard M. (2014): Industrial potential of polyhydroxyalkanoate bioplastic: a brief review, USURJ University of Saskatchewan Undergraduate Research Journal, https://doi.org/10.32396/usurj.v1i1.55.
5. Chia WY., Tang DYY., Khoo KS., Lup ANK., Chew KW. (2020): Nature's fight against plastic pollution:Algae for plastic biodegradation and bioplastic production, Environmental Science and Ecotechnology, 4:100065.
6. Chodak I. (2008): Polyhydroxyalkanoates: origin, properties and applications, Monomers Polymers and Composites from Renewable Resources. https://doi.org/10.1016/B978-0-08-045316-3.00022-3.
7. Dalal J., Sarma PM., Lavania M., Mandal AK., Lal B. (2010): Evaluation of bacterial strains isolated from oil-contaminated soil for production of polyhydroxyalkanoic acids (PHA), Pedobiologia, 54(1): 25–30.
8. De Philippis R., Ena A., Guastini M., Sili C., Vincenzini M.(1992): Factors affecting poly-β-hydroxybutyrate accumulation in cyanobacteria and in purple non-sulfur bacteria, FEMS Microbiology Reviews, 103(2–4):187–194. https://doi.org/10.1111/J.1574-6968.1992.TB05837.X.
9. Gasser I., Muller H., Berg G. (2009): Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants, FEMS Microbiology Ecology, 70(1): 142–150. https://doi.org/10.1111/j.1574-6941.2009.00734.x.
10. Ghatnekar MS., Pai JS., Ganesh M. (2002): Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium sp*, Journal of Chemical Technology and Biotechnology, 49(77): 444–448. DOI: 10.1002/jctb.570.abs.
11. Hejazi P., Vasheghani-Farahani E., Yamini Y. (2003): Supercritical fluid disruption of *Ralstonia eutropha* for polyhydroxybutyrate recovery, Biotechnology Progress, 19:1519–1523. DOI: 10.1021/bp034010q.
12. Hou Q., Zhen M., Qian H., Nie Y., Bai X., Xia T., Rehman MLU., Li Q., Ju M. (2021): Upcycling and catalytic degradation of plastic wastes, Cell Reports Physical Science, 2(8):100514.
13. Ibrahim MHA., Steinbüchel A. (2009): Poly (3-hydroxybutyrate) production from glycerol by *Zobellella denitrificans* MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction, Applied and Environmental Microbiology, 75:6222–6231. DOI: 10.1128/AEM.01162-09.

14. Jiang X., Ramsay JA., Ramsay BA. (2006): Acetone extraction of mcl-PHA from *Pseudomonas putida* KT2440, Journal of Microbiological Methods, 67:212–219 DOI: 10.1016/j.mimet.2006.03.015.
15. Lakshman K., Shamala TR. (2006): Extraction of polyhydroxyalkanoate from *Sinorhizobium meliloti* cells using *Microbispora sp*. culture and its enzymes, Enzyme and Microbial Technology, 39: 1471–1475. DOI: 10.1016/j.enzmictec..03.037.
16. Lemoigne M. (1926): Produits de deshydratation et de polymerization de l'acide β oxybutyrique, Bulletin de Societe Chimique et Biologique, 8:770-782.
17. Li Z., Yang J., Loh, X. (2016): Polyhydroxyalkanoates: opening doors for a sustainable future, NPG Asia Materials, 8: e265, https://doi.org/10.1038/am.2016.48.
18. Muigano MN., Anami SE., Onguso JM., Omare GM. (2023): The Isolation, Screening, and Characterization of Polyhydroxyalkanoate-Producing Bacteria from Hypersaline Lakes in Kenya, Bacteria, 2(2):81-97. https://doi.org/10.3390/bacteria2020007.
19. Muneer F., Rasul Ijaz., Azeem F., Zubair M., Nadeem H. (2020): Microbial Polyhydroxyalkanoates (PHAs): Efficient Replacement of Synthetic Polymers. Journal of Polymers and the Environment, https://doi.org/10.1007/s10924-020-01772-1.
20. Nagalakshmaiah M., Afrin S., Malladi RP., Elkoun S., Robert M., Ansari MA., Svedberg A., Karim Z. (2019): Biocomposites: Present trends and challenges for the future, Book: Green Composites for Automotive Applications, Elsevier, 197-215. 10.1016/B978-0-08-102177-4.00009-4.
21. Panda B., Mallick N. (2007): Enhanced poly-β-hydroxybutyrate accumulation in a unicellular cyanobacterium, *Synechocystis sp.* PCC 6803. Letters in Applied Microbiology, 44(2): 194–198. https://doi.org/10.1111/j.1472-765x.2006.02048.x.
22. Panda B., Sharma L. and Mallick N. (2005). Poly-β-hydroxybutyrate accumulation in *Nostoc muscorum* and *Spirulina platensis* under phosphate limitation, Journal of Plant Physiology, 162(12): 1376–1379. doi: 10.1016/j.jplph.2005.05.002.
23. Prieto MA., Bühler B., Jung K., Witholt B., Birgit K. (1999): PhaF, a Polyhydroxyalkanoate-Granule-Associated Protein of *Pseudomonas oleovorans* GPo1 Involved in the Regulatory Expression System for *pha* Genes, Journal of Bacteriology, 181(3):858–868. https://doi.org/10.1128%2Fjb.181.3.858-868.1999.
24. Ragaert P., Buntinx M., Maes C., Vanheusden C., Peeters R., Wang S., Cardon, L. (2019): Polyhydroxyalkanoates for food packaging applications, Reference module in food science, https://doi.org/10.1016/b978-0-08-100596-5.22502-x.
25. Ray S., Kalia VC. (2017): Biomedical Applications of Polyhydroxyalkanoates, Indian Journal of Microbiology 57(3):261-269. doi: 10.1007/s12088-017-0651-7.
26. Sabirova JS., M. Ferrer M., Lünsdorf H., Wray V., Kalscheuer R., Steinbüchel A., Timmis KN., Golyshin PN. (2006): Mutation in a "tesB-like" hydroxyacyl-coenzyme A-specific thioesterase gene causes hyperproduction of extracellular polyhydroxyalkanoates by *Alcanivorax borkumensis* SK2, Journal of Bacteriology, 188(24):8452–8459. https://doi.org/10.1128/JB.01321-06

27. Sehgal R., Gupta R. (2020): Polyhydroxyalkanoate and its efficient production: an eco-friendly approach towards development, Biotechnology, 10(12):549. doi: 10.1007/s13205-020-02550-5.
28. Shabina M., Afzal M., Hameed S. (2015): Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: Production, biocompatibility, biodegradation, physical properties and applications, Green Chemistry Letters and Reviews, 8:3-4, 56-77, doi: 10.1080/17518253.2015.1109715.
29. Shen L., Hu H., Ji H., Zhang C., He N., Li Q., Wang Y. (2015): Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from excess activated sludge as a promising substitute of pure culture, Bioresource Technology, 189: 236-242.
30. Silva LF., Taciro MK., Carter JM. (2004): Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate, Journal of Industrial Microbiology and Biotechnology, 31(6): 245–254.
31. Singh Saharan B., Grewal A., Kumar P. (2014): Biotechnological Production of Polyhydroxyalkanoates: A Review on Trends and Latest Developments, Chinese Journal of Biology, 802984. https://doi.org/10.1155/2014/802984.
32. Tripathi AD., Yadav A., Jha A., Srivastava SK. (2012): Utilizing of sugar refinery waste (cane molasses) for production of bio-plastic under submerged fermentation process, Journal of Polymers and the Environment, 20 (2): 446–453. DOI 10.1007/s10924-011-0394-1.
33. Tsuge T., Yano K., Imazu S., Numata K., Kikkawa Y., Abe H., Taguchi S., Doi Y. (2005): Biosynthesis of Polyhydroxyalkanoate (PHA) Copolymer from Fructose Using Wild-type and Laboratory Evolved PHA Synthases, Macromolecular Bioscience, 5:112-117.
34. Verlinden RA., Hill DJ., Kenward MA., Williams CD., Radecka I. (2007): Bacterial synthesis of biodegradable polyhydroxyalkanoates, Journal of Applied Microbiology, 102(6):1437-49. doi: 10.1111/j.1365-2672.2007.03335.x. PMID: 17578408.
35. Wallen LL., Rohwedder WK. (1974): Poly-β-hydroxyalkanoates from activated sludge, Environmental Science and Technology, 8(6): 576-579.
36. Wang Y., Guo-Qiang C. (2017): Polyhydroxyalkanoates: Sustainability, Production, and Industrialization. 10.1002/9783527340200.ch2.
37. Zhang J., Shishatskaya EI., Volova TG., da Silva LF., Chen GQ. (2018): Polyhydroxyalkanoates (PHA) for therapeutic applications, Materials Science and Engineering C, Materials for Biological Applications, 1(86):144-150. doi: 10.1016/j.msec.2017.12.035.
38. Zhang K., Hamidian AH., Tubić A., Zhang Y., Fang JKH., Wu C., Lam PKS. (2021): Understanding plastic degradation and microplastic formation in the environment: A review, Environmental Pollution, 1(274):116554. doi: 10.1016/j.envpol.2021.116554. Epub 2021 Jan 23.

AN OVERVIEW OF BACTERIA

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Introduction:

Micro-organisms and their activities are vitally important to virtually all processes on Earth. Micro-organisms matter because they affect every aspect of our lives – they are in us, on us and around us. Microbiology is the study of all living organisms that are too small to be non-visible with the naked eye. This includes bacteria, archaea, viruses, fungi, prions (The term Prion means proteinaceous infectious particles. Prions are the infectious agents responsible for several neurodegenerative diseases in mammals, like, Creutzfeldt Jakob disease. This happens due to the abnormal folding of the proteins in the brain), protozoa and algae, collectively known as 'microbes'. These microbes play key roles in nutrient cycling, biodegradation/biodeterioration, climate change, food spoilage, the cause and control of disease, and biotechnology. Thanks to their versatility, microbes can be put to work in many ways: making life-saving drugs, the manufacture of biofuels, cleaning up pollution, and producing/processing food and drink.

Microbiologists study microbes, and some of the most important discoveries that have underpinned modern society have resulted from the research of famous microbiologists, such as Jenner and his vaccine against smallpox, Fleming and the discovery of penicillin, Marshall and the identification of the link between *Helicobacter pylori* infection and stomach ulcers, and zur Hausen, who identified the link between papilloma virus and cervical cancer. Microbiology research has been, and continues to be, central to meeting many of the current global aspirations and challenges, such as maintaining food, water and energy security for a healthy population on a habitable earth. Microbiology research will also help to answer big questions such as 'how diverse is life on Earth?', and 'does life exist elsewhere in the Universe'?

Bacteria:

Bacteria are the simplest prokaryotic unicellular organisms; highly adaptable and can survive in extremes of temperature, pH, oxygen tension, osmotic and atmospheric pressures. So they are found in almost all natural environments. They can directly or indirectly affect us. A Dutch cloth merchant A. V. Leeuwenhoek (1632-1723) was the first to give the clear idea and description about the microorganisms with the help of microscope. Bacteria share the characters of both plants and animals; therefore they could not be placed in either of these kingdoms. They are placed in a separate class Schizomycetes. Ernest Haeckel (German biologist) proposed a third kingdom protista (1894) with two large groups i.e. as lower protista (bacteria and blue green algae) and higher protista. (Protozoa, algae and fungi).

Characteristics of bacteria:

The bacteria are microscopic, prokaryotic living organisms; considered as the first primitive organisms on the earth. They show following characters:-

1. Bacteria are found in all possible habitats on the earth because of prokaryotic cell nature and are omnipresent.
2. Mode of nutrition: most of the bacteria have heterotrophic - obtain their food directly from the host and so parasitic, some are saprophytic where as some are symbiotic. Some of the bacteria are autotrophic mode of nutrition since they have bacteriochlorophyll (a photosynthetic pigment.)
3. They are unicellular and simple in structural morphology.
4. The cell wall of bacteria is rigid and made up of two types of polymers, i.e. amino acid and saccharide subunits. Cellulose is absent in bacterial cell wall.
5. A well organized nuclear membrane and chromosomes are absent but the DNA strand is present instead of chromosome.
6. In photosynthetic bacteria, chlorophyll pigments are located within cytoplasmic membranes, plastids are absents.
7. Mitochondria are absent but their function is taken over by mesosomes (infoldings of the cell membrane)
8. The cell organelles like endoplasmic reticulum and Golgi apparatus are absent, but ribosomes are abundant.
9. Vegetative/asexual reproduction takes place by binary fission. Sexual reproduction is absent however recombination of genetic material occurs by conjugation, transformation and transduction.
10. The motile bacteria may have one or more flagella made up of eight parallel chains of flagellin (a protein) molecules. Minute hair like cytoplasmic appendages pili emerge through the cell wall. (Composed of a protein - fimbrillin.)
11. Bacteria may take Gram stain (Gram +ve) or may not (gram –ve) and they may grow as aerobic or anaerobic.

Size of bacteria:

Bacteria are very small, microscopic organisms (visible under the light microscope.) Their size is variable (0.75 µm to 1.5 µm), an average of each bacterial cell ranges from 0.5 µm to 2.0 µm in diameter. The smallest size of bacteria is about 0.1 µm whereas largest size of bacteria is 60 x 6 µm in diameter. Generally the size of various bacteria is variable- For example 0.5 to 2.5 µm, (Spherical / *Coccus*), 0.3 to 1.5 µm, (Rod shaped /*Bacillus*) 1 µm x 1.5 µm (Spiral / *Spirillum*). A single drop of water may contain about 50 billions of bacteria.

Forms of bacteria (Shapes):

The bacterial cells show considerable variation in their shape. Morphologically bacteria have been classified in to three main forms. i. e. A) Spherical (*Coccus)* B) Rod shaped (*Bacillus*) and C) Spiral (helical) bacteria

A) Coccus (spherical) bacteria:

These are the simplest forms of bacteria which appear like a sphere. (Cocci -a Greek word kokkos – grain) They may be ellipsoidal, bean shaped and lanceolate. They measure 0.5 to 1.25 µm in diameter; they are non-motile, nonflagellated and occur in variable size and shape. On the basis of their arrangement, cell division, the number of cells in clusters and biological properties, they are classified into the following six groups (Figure 1).

1. **Micrococci:** When bacterial cells occur singly or irregularly. They are saprophytes (in water and air) e.g. *Micrococcus agilis*,
2. **Diplococci (Dipos - double):** When bacteria occur in pairs and divide in one plane. e.g- *Meningococcus*
3. **Streptococci (Gk. Streptos - curved, kokkos- berry):** When bacteria occur in long chain and divide in one plane. e.g. *Streptococcus lactis*.
4. **Tetracocci (Gk. Tetra- four):** When they occur in a group of four cells and divide in two planes at right angle to one another. e.g. *Pedicoccus cerevisiae*
5. **Staphylococci (Gk. Staphyle- cluster):** An irregular group of many spherical bacterial cells, they divide in several planes resulting in irregular bunches of cells, appear like a clusters of grapes. e.g. *Staphyllococcus albus* -
6. **Sarcinae (L. sarcio to tie):** When spherical bacteria divide in three planes at a right angle to one another in a regular pattern producing a cuboidal arrangement of cells (packet of 8, - more cells.) e.g. *Sarcinae lutea*

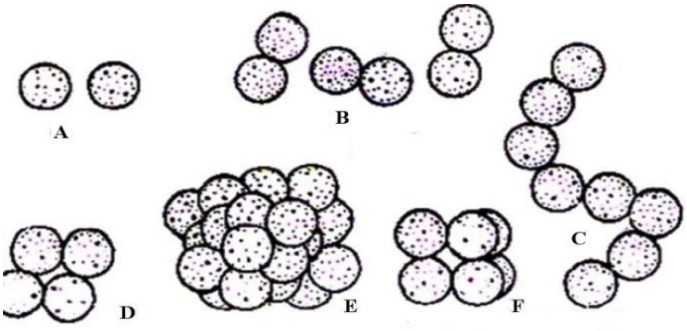

Figure 1: Different type of coccus bacteria
A-*Micrococcus*, B-*Diplococcus*, C-*Streptococcus* D-*Tetracoccus*
E- *Staphylococcus* F- *Sarcina*

B) Bacillus (rod) shaped bacteria

They are very common form of bacteria, they are rod-shaped, cylindrical or elongated, motile or non motile in nature, they may be straight or like cigarette or curved. An average *Bacillus* (Latin word bacillium- a stick or a rod.) is about 1.5 µm x 0.5 µm in size. These are non spore forming and are responsible for paratyphoids, desentry, diphtheria and tuberculosis etc. (e.g. *Bacillus anthrasis, B. fastidious, B. polymyxa* and *Lactobacillus.)*

On the basis of their arrangement, the *Bacillus* bacteria are classified into three groups which are as follows.

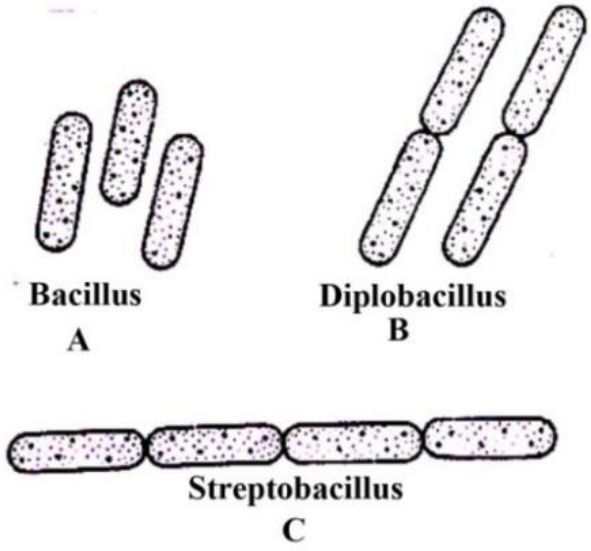

Figure 2: Different types of bacillus bacteria.

1. **Bacilli:** When bacteria occur as isolated rods
2. **Diplobacillus:** When bacillus bacteria occurring in pairs e.g. *Corynebacterium dikphtheriae* (Fig-8-B)
3. **Streptobacilli:** The bacilli bacteria of this group occur in long chains e.g. *Bacillus tuberculosis*

C) Spiral (helical) bacteria

The helical forms are known as vibrios or spirilla. (The Latin word- spiral – coil). They appear like a cork screw. These are the slightly larger and elongated spiral rods. A *Spirillum* has more than one turn of a helix and it is about 1.5 µm x 15 µm in size. These bacteria have one or more flagella at each pole. They usually occur singly or in small chains or rarely in groups. e.g. *S. volutans.* Besides the above mentioned, there are following major morphological groups of bacteria occur in the following shapes.

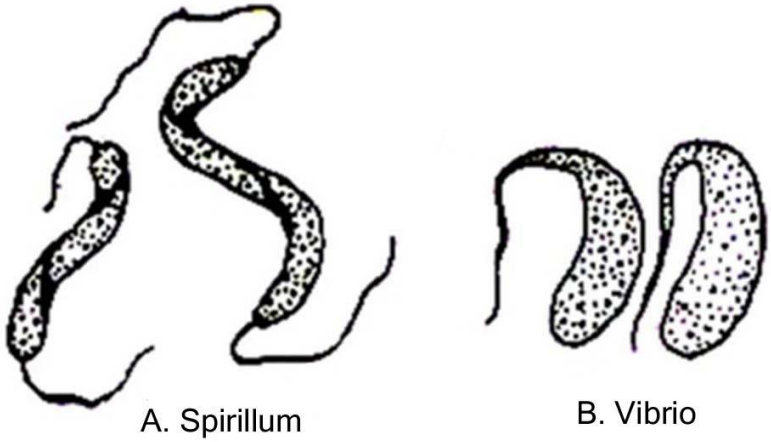

Figure 3: A- Spirillum, B- Vibrio

1. Spirilla: (L. spira- coil). These are coiled forms of bacteria showing twists with one or more turns. e.g. *Spirillum minu* (pathogenic) transmitted through the bite of rats

2. Vibrios: (L. Vibrio - vibrate). These are the slightly curved rods (fresh water forms) of half turn. They appear like the comma sign (❟) and are commonly known as comma bacteria. They are monotrichous (i.e.-with single flagellum at the tip) and -10 µm x 1.5 to 1.7 µm in size. e. g. *Vibrio coli* and *V. cholera*.

3. Filamentous: Some bacteria like *Beggiatoa* and *Thiothrix* appear like filamentous.

4. Pleomorphic: Some bacteria are capable of changing their shape and size temporarily in response to changes in the environmental conditions. e. g. *Acetobacter* may occur as *Bacillus* (single rods).

Ultra structure of bacteria cell:

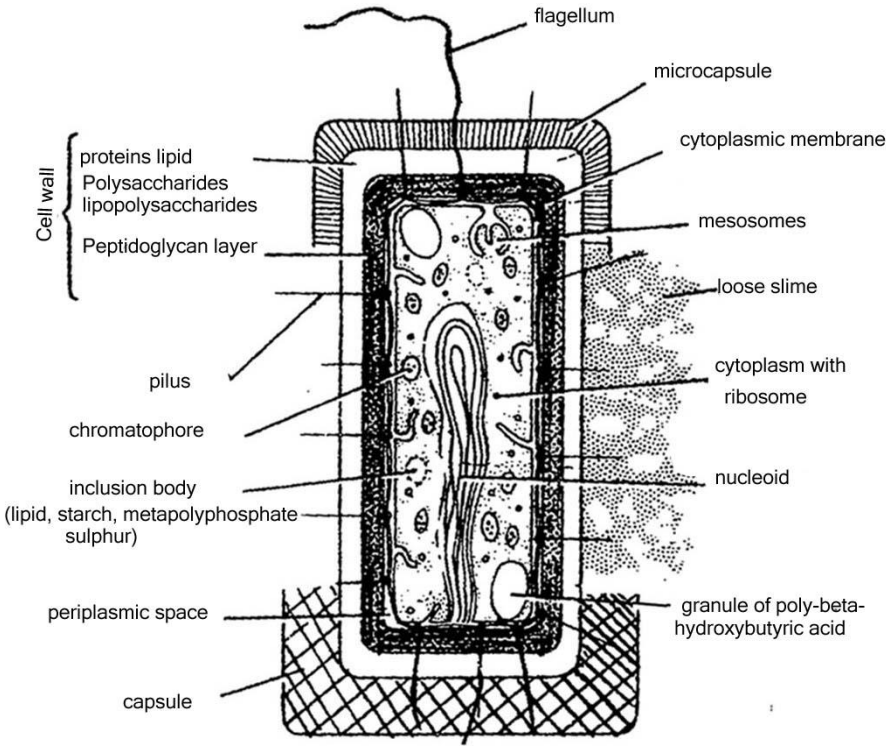

Figure 4: Different parts of bacterial cell seen under electron microscope

Ultra structure can be studied better cytologically with the help of electron microscope and staining techniques. The bacterial cell shows a typical prokaryotic structure, the ultra structure can be studied under two headings.

A) Protectie layers and

B) Cytoplasm and its inclusions.

A) Protective layers

I) the outermost layer (slime layer)

II) the middle layer (cell wall) and

III) The innermost layer (cytoplasmic membrane.)

I) Slime layer (Outer layer)

It is present on the outer most surface of the cell wall, made up of polysaccharides (i.e. dextran, dextrin, lavan) and or polypeptide chains of amino acids. When it is is made up of only polysaccharides, it forms a viscous layer which is called as slime layer but when it is composed

of amino acids along with polysaccharides then it is known as capsule (Glyocalyx) as shown in figure 4.

This layer protects the cell from desiccation and antibodies. The capsulated bacteria also remain unaffected by phagocytosis.

II) Cell wall (Middle layer):

In bacteria; the cell wall has a granular structure which is a tough, rigid and without cellulose and hemicellulose. The cell wall is variable in thickness (50 to 100 A^o). It is composed of N-acetyl glucosamine, N-acetyl muramic acid and a peptide chain of 4-5 amino acids. These three constitents together form a polymer known as peptidoglycan or mucopeptide. Some other chemicals, like teichoic acid, protein, polysaccharides, lipoproteins and lipopolysaccharides are also present in the cell wall. The rigidity of bacterial cell wall is due to this polymer.

The function of the bacterial cell wall is to give the mechanical support, shape and rigidity. The cell wall can withstand with the osmotic pressure of about eight atmospheres per square centimeter.

Staining of bacteria (Gram's stain):

A simple staining procedure was discovered by a Danish Physician, Christian Gram in 1884. The group of bacteria which retains the stain even after decolourization with alcohol is known as Gram positive whereas those lose stain after treatment with alcohol are called as Gram negative e.g. all spore (endospore) forming bacteria are Gram positive and all flagellated forms are Gram negative. The second method is acid fast stain. Due to the presence of waxy or fatty material on the surface of some bacteria, they cannot be stained by simple techniques but can be stained by Ziehl-Neilson technique e.g. *Mycobacterium tuberculosis* and *M. leprae*.

III) Cytoplasmic membrane (Inner layer):

A semipermeable cytoplasmic membrane about 75 A^0 in thick, lies below the cell wall. Chemically it is composed of a double layer of phospholipid molecules, which are of two types i.e. hydrophobic and hydrophilic. The hydrophilic phospholipid molecules are present towards the outer side and the hydrophobic towards the inner side. Proteins are found embedded in the lipid layer. The membrane is rich in sterols which accounts for the resistance of bacteria to antibiotics.

This layer is rich in enzymes of various metabolic pathways such as synthesis of lipopolysaccharides; phospholipids, teichoic acid etc. It also controls the entry of organic and inorganic nutrients in the cell.

Mesosomes:

These are the localized infoldings of the cytoplasmic membrane, there may be 2-4 mesosomes in a cell and the number is usually higher in bacteria which show high respiratory activity – e.g. nitrifying bacteria. It has been suggested that the highly infolded membrane system of mesosomes serve to be centre of respiration but absence of enzymes like ATPase, dehydrogenase and cytochrome in mesosomes, indicates that they are not sites of respiration. They probably participate in the formation of septum during cell division (Fig. 5).

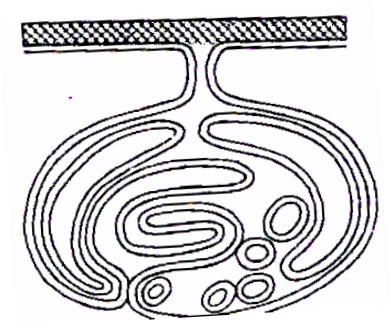

Figure 5: Bacterial: Mesosome

B) Cytoplasm and its inclusions:

The cytoplasm is a complex mixture of carbohydrates, proteins, lipids, minerals, nucleic acids and water. It stores organic material like glycogen, volutin and poly-β-hydroxybutyrate. Some photosynthetic and non-photosynthetic bacteria also accumulate sulphur and iron in their cytoplasm. The cytoplasm also contains a chromatic or nuclear area. The cell organelles like mitochondria, endoplasmic reticulum, centrosomes and Golgi complex are absent in bacteria, but in the photosynthetic bacteria, chromatophores are found in cytoplasm.

i) Nuclear material: Basophilic property of bacterial cells is due to the abundance of ribosomes in their cytoplasm. So to stain the bacterial nucleus, selectively fixed cells are first treated with ribonuclease or HCl which hydrolyse the ribosomal RNA. The bacterial nucleus is without nuclear membrane, nucleolus, chromonemata and nuclear sap. Such type of nucleus is known as nucleoid (genophore). The nucleoid appears to be fibrillar and composed of a double stranded DNA (1000 long µm) with $5x10^{6}$ base pairs and a mol. wt. of about $3x10^{6}$ Daltons. The DNA molecule is usually forms ring like structure or sometimes remains diffused throughout the cytoplasm. The bacterial DNA is devoid of histones hence can not be compared with the chromosomes of eukaryotic cells. The DNA may be double or single stranded (Fig. 6 A, B).

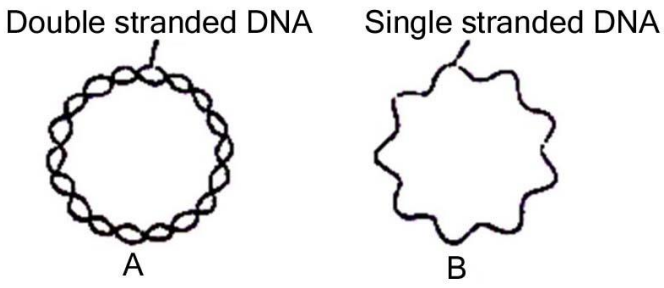

Figure 6: Bacterial DNA: A-Double stranded, B-Single stranded.

ii) Plasmids: Bacterial cells also contain some extra-chromosomal hereditary determinants which are either independent of bacterial chromosomes or are integrated into it. Lederberg (1952) was the first who coined the term plasmids for extra chromosomal hereditary determinants. Plasmids carry only non-essential genes and have no role in viability and growth of bacteria; hence they are also defined as dispensable autonomous elements, e.g. F-factor (F-

fertility) which determines the maleness in bacteria. It is an autonomous element separate from the bacterial chromosome. It is transmitted by cell to cell contact or by external agencies (Fig. 7).

Plasmids have been classified on the basis of the host properties. There are nine plasmids are Cryptic plasmids, penicillinase plasmids Tumor inducing plasmids, Mercury resistant plasmids, plasmids of *Pseudomonas*, Col-factor, plasmids of the Gram negative bacteria, R-factor (for resistance) plasmids, F-factor (for fertility) plasmids.

Plasmids are the circular double stranded DNA molecules. Each plasmid may contain as many as 100 genes. The plasmids can replicate similar to that of chromosome replication under their own genetically determined system.

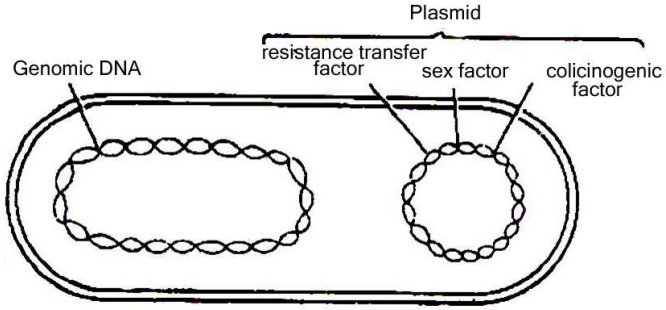

Figure 7: Bacteria: Genome and plasmid of a bacterial cell

iii) Ribosomes: Ribosomes are the sites of protein synthesis in bacterial cells. In bacteria, the ribosomes are free in the cytoplasm; their number varies from 10,000 to 15,000 in a cell. Bacterial ribosomes are of 70s type and consists of two subunits. The sedimentation constant of the larger subunits is 50s molecules (RNA with 35 amino acids) and that of the smaller subunits is 30s. In a young bacterium, ribosomes may occur in groups of 4-6 or more, they are held together by special RNA molecules known as messenger RNA. Such type of group of bacteria is known as polyribosomes.

Out growths of the cell wall:

Flagella: The locomotion of bacteria takes place by the presence of thin flagella. Each flagellum is a whip like structure of almost uniform thickness, arises from the cytoplasm. They are composed of several chains of flagellin (a protein- 40 A^0 diam.) molecules forming a more or less cylindrical filament (9+2 fibrillar structure). The length of flagella is about 4-5 µm long, and diam is about 120-150 $A^{0,}$ and are concerned with movement. The bacteria are classified in to two groups on the basis of presence or absence of flagella - i. e. **Atrichous** The bacteria are devoid of flagella, (non-motile.) e. g. *Lactobacillus* (Fig. 8) **and Trichous-** The bacteria with flagella (motile).

According to their number, position and arrangenment, the trichous bacteria can be classified in to following groups.

a) Polar flagellum It is found in Gram-negative bacilli and spirilla. The bacteria can be recognized as fallows

i) Monotrichous: When only a single flagellum is present at one end of the bacterial cell. *Vibrio cholerae* (Fig. 8)

ii) Amphitrichous: When bacterial cell shows the presence one or more flagella at both the ends. e.g. *Nitrosomonas*.

iii) Cephalotrichous: The bacterial cells have two or more flagella at one end only. e.g. *Pseudomonas fluorescens*.

iv) Lophotrichous: When bacteria which have a group of flagella at one or both the ends. ex. *Spirillum volutans*.

b) Peritrichous: (**Non-polar flagellation)-** In petrichous or non-polar flagellation, the flagella are eventually distributed throughout the surface of the cell ex. *Bacillus typhosus* and *Clostridium*.

Pili or fimbriae (Fig. 9): Many Gram-negative bacteria have minute rigid cylindrical appendages extending outwards from the cell wall. These are known as pili or fimbriae. They are smaller than flagella (2 µm long x 30-50 A^0 diameter) and their number is usually several hundred, they are composed of protein are known as pilins. Pili are not the organelles of locomotion but their general function is to confer the adhesiveness. In addition to normal pili there are some special types of pili known as sex pili. There are usually 1-5 sex pili per cell. They are helful in the transfer of genetic material.

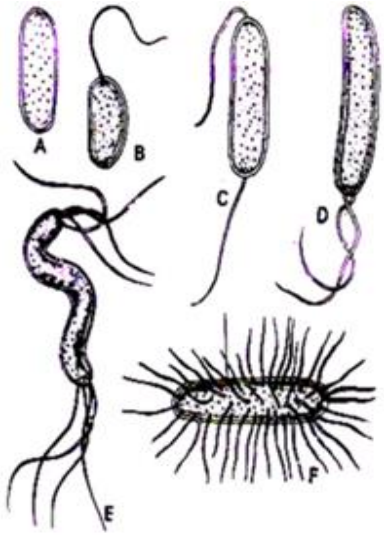

Figure 8: A-F Bacteria: A- Atrichous, B- Monotrichous, C- Amphitrichous, D- cephalotrichous, E- Lophotrichous, F- peritrichous

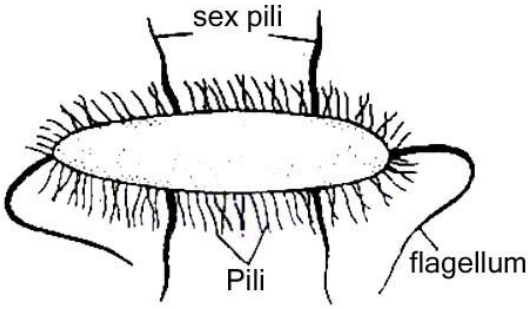

Figure 9: Bacterial cell: Pollar flagella and pili

References:

1. Campbell, N.A., Reece J.B., Urry L.A., Cain M.L., Wasserman S.A. Minorsky P.V., Jackson, Microbiology.
2. Jackson, R.B. (2008). Biology, Pearson Benjamin Cummings, USA. 8th edition.
3. Pelczar, M.J. (2001). Microbiology, 5th edition, Tata Mc Graw-Hill Co, New Delhi.

PRESENT STATUS OF NANOTECHNOLOGY IN AGRICULTURE AND FOOD SECTOR

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Abstract:

Agriculture and plant biotechnologists have recently begun to pay attention to the potential of nanotechnology in agriculture and food biotechnology. Although the usage of nanotechnology in the fields of physics and chemistry is well recognised and numerous products have been produced using this technology are now available on the market, and the use of nanotechnology in the food and agriculture sectors is relatively new and therefore, not as well known because it is new. USDA, the United States Department of Agriculture produced a roadmap for the first time in order to solve this issue, which was published in September 2003. It was anticipated that nanotechnology would change how crops are grown and how food is produced throughout the whole agriculture and food industries.

Keywords: Nanotechnology, Agriculture, Biotechnology, Physics, Chemistry, Food industries.

Nanotechnology:

The term "nanotechnology" (derived from "nano," which in Greek means "dwarf") is typically used to describe a technology that uses materials with a nm range of 0.1 to 100m) in size. Consequently, the primary goal of nanotechnology focuses on unique characteristics of developing materials from their nanoscale scale. These items, which are brand-new or significantly improved various characteristics are developed either by a top-down strategy which entails lowering the tiniest structures down to the nanoscale, such as photonics applications in nano-electronics and nano-engineering.

Potential of nanotechnology

- The promise of nanotechnology in the sectors of textiles, information technology, health care, and the sectors of communication technology and energy sectors is well known.
- In fact, nanotechnology play a major role in anti-bacterial dressings, transparent sunscreen lotions, stain resistant fabrics ans scratch free paints.

Nanotechnology in agriculture

Nanotechnology in agriculture has the potential to alter the food sector withm novel instruments for a range of uses, such as the following:

(i) Plant protection (disease detection for defence of crops against various illnesses); for clever sensors and smart delivery for this objective Systems are being created that will assist the using the agricultural sector to fight viruses and other crop pathogens

(ii) Increasing the water use efficiency (WUE); Nutrient use efficiency (NUE)

(iii) Improved pesticide effectiveness and herbicides using nano-structured materials using lesser doses is possible thanks to analysis.

(iv) Protection of environment indirectly through the use of alternative energy supplies.

Nanotechnology in precision farming

Precision farming, which is presently becoming more and more popular all over the world, is another application of nanotechnology. We will first define precision farming and then go through how nanotechnology is applied in this practise.

The practise of collecting data on the geographical and temporal variation within a field is known as precision farming. Precision farming is a system that maximises output (i.e. crop yields) with minimal input (i.e. fertilisers, pesticides, herbicides, etc.). This information is then utilised to manage inputs and techniques in farming in such a manner that one obtains minimum output with minimum input. Monitoring environmental factors and taking targeted measures help achieve this. Precision farming uses computers, global positioning systems (satellites), and remote sensing equipment to measure extremely localised environmental variables. In order for a crop to grow as efficiently as possible, this enables the identification of the type and location of problems.

GPS for precise agriculture, Farming was practised in the area under the presumption that a field is homogeneous. By segmenting the field into smaller zones and controlling these zones independently, modern agricultural management approaches are evolving to account for field variability. The agricultural community uses the Global Positioning System (GPS) extensively for this purpose. An accurate location may be determined at any time, in any weather, and from any location on Earth thanks to the space-based global positioning system, or GPS. Anyone with a GPS receiver device can freely access it because it is maintained by the US government.

The GPS device is composed mostly of three segments: the space segment, the control segment, and the user segment. The constellation of 24 operational and 3 spare satellites orbiting the Earth serves as the foundation for the space section. The master control facility for the control segment is located in Colorado, and it consists of a system of five monitoring stations spread out around the globe (USA). The user segment, which consists of GPS receivers and is used in precision farming, is the one that is expanding at the highest rate. which translate the signals from the satellites into position, velocity, and time. Precision farming is one of the many uses for this data, which also includes navigation, positioning, time dissemination, and research.

GPS receivers and nanosensors for precision agriculture. GPS receivers are made possible by the fabrication of incredibly small sensors and monitoring systems thanks to nanotechnology. Future techniques for precision farming will be impacted by this. The rising usage of autonomous sensors connected to a global positioning system (GPS) for real-time monitoring will be one of the key functions for nanotechnology-enabled gadgets (as discussed above). These nanosensors might be dispersed around the field to track crop development and soil quality. Already, several regions of the USA and Australia use wireless sensors. For instance, the IT firm Accenture assisted one of the Californian vineyards, Pickberry, in Sonoma County with the installation of wifi equipment. The fact that such a system makes it possible to

grow the best grapes, which in turn results in finer wines produced to be sold at a premium price, justifies the initial cost of setting it up. Of course, wireless networks like this are not just used in vineyards.

The combination of nanotechnology and biotechnology in sensors will result in a number of devices with improved sensitivity, enabling a quicker reaction to environmental changes. In the end, precision farming with the aid of smart sensors enables increased agricultural productivity by delivering precise information and assisting farmers in making better decisions.
The following are some examples of how GPS is used in farming: I mapping yields (GPS + combine yield monitor); (ii) mapping variable rate planting; (iii) suggesting variable optimum fertiliser application; (iv) suggesting variable optimum pesticide application; (v) field mapping for records and insurance purposes (GPS + mapping software); and (vi) parallel swathing (GPS + navigation too).

Nanotechnology in food industry

Nano-foods

Throughout the first ten years of the twenty-first century, the effects of nanotechnology on the food business have been more obvious. Better and safer food is currently being prioritised more. A number of businesses that were reticent to publicise their nano-food research programmes have recently done so and announced their plans to enhance current products and create new ones in order to preserve market supremacy.

Foods that are produced, processed, or packaged utilising methods and equipment based on nanotechnology are referred to as nano-those rather than foods that have been atomically altered or those created by nanomachines. Although ambitious ideas of producing molecular food with nanotechnology exist, this is not possible in the near future. Instead, nanotechnologists are more upbeat about how nanotechnology could alter the current food processing system, guarantee the safety of food items, and promote a healthy food culture. They also believe that some additives will improve the body's ability to digest and absorb food as well as the nutritional quality of food. Although some of these objectives might take some time, nanotechnology is already used in the food packaging business.

Packaging and food safety

Many businesses have set out to create intelligent packaging that can detect minor tears or holes, respond to changes in temperature and moisture, and inform the consumer if the food is tainted in order to maximise product shelf life. Solutions such as

(i) Altering the permeation behaviour of foils
(ii) Enhancing barrier properties (mechanical, thermal, chemical, and microbial)
(iii) Enhancing mechanical and heat-resistance properties
(iv) Developing active antimicrobial and antifungal surfaces
(v) Sensing as well as significant microbiological and biochemical changes can all be achieved with the help of nanotechnology.

Food contamination detection may potentially benefit from advances in nanotechnology in terms of sensitivity or simplicity of use. The Nano-Bioluminescence Detection Spray, for

instance, was created by AgroMicron and contains a luminous protein that has been tailored to adhere to the surface of microorganisms like *Salmonella syphimurium* and *E. coli.* When bound, it creates a bright glow that makes it simple to spot tainted food and drinks. The level of bacterial contamination increases with the intensity of the illumination. The company was developing novel spray techniques to use in ocean freight containerized shipping as well as to combat bioterrorism and plans to market the product under the name BioMark. sensors for 'good food,' A transportable nanosensor has been created by EU researchers working on the Good Food Project to identify chemicals, diseases, and poisons in food. This eliminates the need to ship samples to laboratories, which is expensive and time-consuming, and enables food to be examined for safety and quality at the farm, during transportation, during processing, or at the packing facility. The group is also creating a tool to find infections using DNA biochips. This method might also be used to check for the presence of other dangerous bacteria in meat or fish, or fungi that influence fruit. Microarray sensors that can be used to detect pesticides on fruits and vegetables as well as those that will monitor the farm's environmental conditions are also being developed as part of the project. They are referred to as "Good Food Sensors."

Nanosensors in the food industry, Honeywell, a multinational technology RandD business, is also using tiny nanosensors to keep an eye on Minnesota's grocery stores. The use of technology is assisting store owners in identifying foods that have expired and serving as a reminder to place a new purchase order. By 2010, it is expected that the market for wireless sensors would be worth $7 billion.

Food processing: nanocapsules/ nanoparticles for functional/interactive foods

Nanotechnology is already having an impact on the creation of interactive or functional foods, which can supply nutrients more effectively and adapt to the body's needs in addition to packaging. A number of research teams are also aiming to create novel "on demand foods," which will persist in the body and feed cells when they require nutrients. The creation of nanocapsules that can be used in food processing, such as adding nanoparticles to already-existing foods to boost nutritional absorption, is a crucial component of this industry. Here are a few instances.

Bread that is "Tip-Top" Up for fish oil One of Western Australia's finest bakers has been successful in adding nanocapsules containing tuna fish oil, a source of omega 3 fatty acids, to their best-selling product, "Tip-Top" Up bread. The fish oil won't taste bad because the microcapsules are made to only split apart after they've reached the stomach.

Nano-sized Self-assembled Liquid Structures (NSSL) technology is used by the Israeli company Nutralease to provide nutrients to cells in the form of tiny particles. The particles, which have an average diameter of 30 nm and are expanded micelles (hollow spheres comprised of fat with an aqueous inside), are micelles. The watery interior holds the nutrients, or "nutraceuticals." The carriers now contain lycopene, beta-carotene, lucein, phytosterols, CoQ10, and DHA/EPA as well as other nutraceuticals. The Nutralease particles make it easier for these substances to pass from the gut into the bloodstream, boosting their bioavailability. Shemen

Industries has already marketed and used the method to distribute Canola Activa oil, which it claims lowers cholesterol intake by 14% by competing with bile for solubilization.

Present status of nanotechnology in the world food market

The new industrial revolution has been dubbed nanotechnology, and both industrialised and emerging nations are looking into it to gain market share. However, the United States now leads with a four-year, 3.7 billion US$ investment through its National Nanotechnology Initiative (NNI), with Japan and the European Union trailing after with significant funding commitments (750 million and 13 billion US$, respectively, per year). Although financing levels in emerging nations may be comparably lower, this has diminished some nations' influence on the global scene. For instance, China's contribution to academic papers on nanoscale scientific and engineering themes increased from 7.5% in 1995 to 18.3% in 2004, moving it up to the second spot in the world from fifth.

With an emphasis on applications particular to the economic growth and needs of their own countries, other developing nations like India, South Korea, Iran, and Thailand are also catching up. Iran, for instance, has a special nanotechnology programme for the food and agriculture sectors. According to a recent estimate, the market for nano-food will grow from 2.6 billion US dollars to 20.4 billion by 2010. According to the study, Asia will have the largest market for nano-food in 2010, with China leading all other nations because it has more than half of the world's population. There are already more than 400 companies engaged in nanotechnology research and development (RandD) worldwide, and during the next ten years, this number is anticipated to reach more than 1000. (2010-2020). In terms of sheer numbers, the USA is in the lead, followed by China, Japan, and the EU According to a prediction, the market for nanotechnology was worth 7.6 billion US dollars in 2003 and will be worth 1 trillion by 2011. However, nanotechnology's full promise in the food and agriculture industries has not yet been fully exploited. India's Use of Nanotechnology in Food and Agriculture (and Potential Contribution to Second Green Revolution)

In the first ten years of the twenty-first century, India began to grasp the significance of nanotechnology research and development. In May 2007, the Government of India's Department of Science and Technology (DST) launched a mission (Nano-Mission) on the Nanomaterials Science and Technology Initiative (NSTI) with an investment of more than Rs. 1000 crores for five years (http://www.dst.gov.in.in/ scientific - programme / sernsti.htm). More than 100 research projects received funding under this programme. These studies focused on the synthesis and construction of DNA chips, nanoporous materials, nanotubes, nanowires, and ceramic nanoparticles. The development of a variety of shared facilities and infrastructure was also supported.

Nanotechnology in agriculture sector in India

It's critical to evaluate the opportunities and obstacles present in order to realise the promise of nanotechnology in Indian agriculture. The following are some of the topics related to Indian agriculture where nanotechnology can offer workable solutions: Plants can utilise nanofertilizers for I a gradual release and efficient use of water and fertiliser, and (ii) controlled

release of pesticides (nanocides). Delivery of nutrients and medications for livestock and fisheries; (iv) nanobrushes and membranes for soil and water purification; (vi) nanobrushes and membranes for cleaning fishponds; and (vii) nanosensors for soil quality and for plant health monitoring; precision agriculture; and controlled environment agriculture.

Nanotechnology in food sector in India

Nanotechnology can also be used in the food processing industry, where nano-composites and nano-biocomposites are employed for a number of functions, such as the following; Anti-microbial nanoemulsions are used to clean food processing equipment, packaging, and plastic film coatings, which are both utilised to package food.

Current status

The overall state of nanotechnology is far better globally than in Asia. According to some estimates, the global market was worth $12 billion. Nanobiotechnology would account for a small portion of this industry in 2008 while expanding at a rate of 24%.

Biotechnology is one of the many sectors where nanotechnology applications are rapidly evolving in the US and Japan. Several Asian nations, including Taiwan, South Korea, Singapore, China, and Japan, are engaged in extensive ongoing research in nanotechnology (including its application to agriculture). Between 2008 and 2010, Asia-Pacific nations spent more than $2 billion in total, and this trend is expected to continue. After the United States, Japan has invested heavily in nanotechnology research and development since the 1980s. Since 2001, their populations have also grown significantly in China, South Korea, and Taiwan. Australia is currently developing a national strategy but has a sizable amount of financing and infrastructure in place. While some products have already reached the commercial stage, India's nanotechnology business is now in its infancy and has not yet developed fully. Some of Delhi University's nano-encapsulation-based products have already been commercialised.

Future trends

Although nanobiotechnology will undoubtedly be employed in medical, its application in agriculture is just slowly expanding. Using nanotechnology to apply pesticides, herbicides, and fertilisers economically is a given. Additionally, it will be used to the packaging sector. The same causes that are driving the expansion of nanobiotechnology around the world also apply to India. As a result of nanotechnology's potential to change numerous industries, including biotechnology, pharmaceuticals, IT, catalysts, and surface coatings, the Indian government is investing significantly in the field. However, the lack of a well defined national nanotechnology policy, a rigid institutional structure, and a dearth of capital support may prove to be a barrier to the nation's long-term advancement in nanoscience and nanotechnology. Nanotechnology has the potential to overtake information technology (IT) and biotechnology as India's third-largest industry by the day that the government implements enticing feed-in tariffs, incentives to industries, and a national policy. Even on a global scale, the application of nanotechnology to agriculture and food systems is in its early stages, and its success will depend on how stakeholders ultimately view it. Applying nanotechnology to this industry also necessitates the creation of robust governance structures and efficient regulatory frameworks with input from all

stakeholders. Only then will nanotechnology bring about India's much-needed second green revolution in the agricultural sector.

References:

1. Aruoja V., Dubourguier H., Kasamets C., and Kahru K.A (2009). Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae, Pseudokirchneriellasubcapitata. Sci. Total Environ., 407,1461-1468.
2. Auffan M., Rose J., Bottero J.Y., Lowry G.V., Jolivet J.P and Wiesner M.R (2009). Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective Nat. Nanotechnol, 4, 634-664.
3. Díaz S., Pérez J., Mateos A., Marinescu M. and Guerra, B. (2012). A novel methodology for the monitoring of 1Precision agricultural production process based on wireless sensor networks. International Journal of Science, Engineering and Technology Research (IJSETR). 7(6), 252-265
4. Finglas P.M., Yada R.Y and Toldra, F. (2014). Nanotechnology infoods, science behind and future perspectives Trends Food Sci and Technol, 40, 125-126.
5. He, X., Deng, H., and Hwang, H. (2019). The current application of nanotechnology in food and agriculture. J. Food Drug Anal. 27, 1-21.
6. Khot L.R., Sankaran S., Maja J.M., Ehsani R., and Schuster E.W(2012). Applications of nanomaterials in agricultural production and crop protection, a review Crop Protect, 35,64-70.
7. Mishra S., Singh A., Keshwani C and Singh H.B (2014). Nanotechnology Exploring potential application in agriculture and its opportunities and Constraints, Biotech Today 4(1) 9-14.
8. Peters R.J., Bouwmeester, H., Gottardo S., Amenta V., Arena M., and Brandhoff P (2016). Nanomaterials for products and application in agriculture, feed and food Trends Food SciTechnol, 54 ,155-164.
9. Sozer, N and Kokini, J.L (2009). Nanotechnology and its applications in the food sector Trends Biotechnol, 27, 82-89.

PGPR AND THEIR BENEFICIAL ATTRIBUTES

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Introduction:

An essential component of the functioning of terrestrial ecosystems is soil, which is a dynamic, living, natural body. It stands for exceptional harmony between various physical, chemical, and biological components. It is a non-renewable resource whose health has an impact on how effectively the environment is managed, how much food is produced, and how the world is balanced. The ecology of soil depends on diverse bacterial genera, which play an important role in the ecosystem's various processes. Because they are the main factors in the recycling of nutrients, they are essential for plant health, plant productivity, and soil fertility. In comparison to the bacteria found in bulk soils, the bacteria found in the rhizosphere or colonising any portion of the plant are more effective and adaptable in carrying out nutrient transformation and mineralization. Agronomists and environmentalists are now placing a higher value on biological methods of enhancing plant health and agricultural productivity as a result of the detrimental effects of widespread chemical fertiliser use. In this context, research is ongoing with a focus on a variety of advantageous rhizobacteria with novel traits like pesticide degradation, heavy metal detoxification, salinity tolerance, and biocontrol of different plant pathogens, as well as plant growth-promoting activities like the production of growth hormones, siderophore (an iron chelator), hydrogen cyanate, ammonia production.

Consequently, numerous symbiotic (Rhizobium, Mesorhizobium, Bradyrhizobium), associative (Azospirillum), and nonsymbiotic (Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azomonas) rhizobacteria are now used globally as plant probiotics to promote plant growth and development under various stresses (Ahemad and Khan, 2011). In this chapter, we will clarify the idea of beneficial rhizobacteria as probiotics, the underlying principles of plant growth stimulation, and the advantages of their inoculation on soil fertility, plant health, and plant productivity.

Rhizosphere

The term "rhizosphere" refers to the area of soil that surrounds a plant's root system and was originally used by Hiltner in 1904. Due to its abundance of bacteria and microbial activity, the rhizosphere is frequently referred to as a "storehouse of microbial activity." A group of beneficial bacteria found in soil are referred to as "rhizobacteria" in several contexts.

Rhizosphere capable of colonising the area around the roots. The plant's roots sustain it mechanically and aid in water and nutrient intake. In addition to this, plant roots produce, store, and exude a wide variety of substances known as root exudates. According to plant species and physiological situation, these exudates' contents vary (Kang *et al.*, 2010). The wide variety of

chemical substances (such as sugars, flavonoids, amino acids, etc.) secreted as root exudates alter the physical and chemical characteristics of soil, which in turn controls the composition of the microbial communities of soil found in the rhizosphere (Dakora and Phillips 2002). Therefore, the rhizo-microbiome's (the bacteria present in the rhizosphere) composition is different from the microbial community of the nearby soil or bulk soil. While some of the substances generated by plant roots serve as deterrents to microorganisms, others serve as attractants to draw them in.

The bacteria and the by-products of them also engage in a range of interactions with plant roots. Plants and microbes can interact in a good, negative, or neutral way. The quantity and quality of root exudates are altered by microbial activity in the rhizosphere, which also influences how plants receive different important nutrients. Such interactions can vary nutrient dynamics, affect a plant's vulnerability to disease and abiotic stress, and affect the growth and development of plants. Crop plants' health and productivity can be enhanced by utilising these advantageous rhizobacteria.

Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria possess the ability to improve plant growth and productivity as they provide various essential nutrients (macro- as well as micronutrients) to plants. They are good candidates to be used as probiotics for plants as they live in close association with plants and possess all the traits of good inoculant. So, PGPR act as probiotic for plants. PGPR have gained considerable interest in research as plant probiotics because they stimulate plant growth, increase crop yield in an environment friendly and sustainable manner, and reduce the cost of chemical fertilizers. The term PGPR was first coined by Kloepper et al. in 1980.The use of PGPR as probiotic for plants is a better way to grow plants with reduced pollution from fertilizers and pesticides

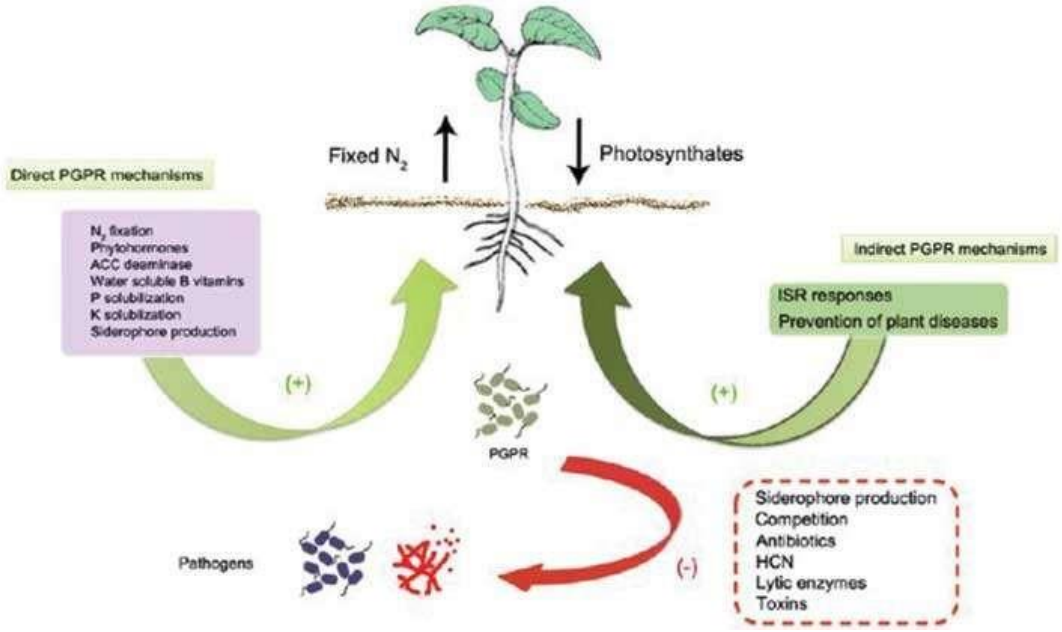
Characteristics of PGPR

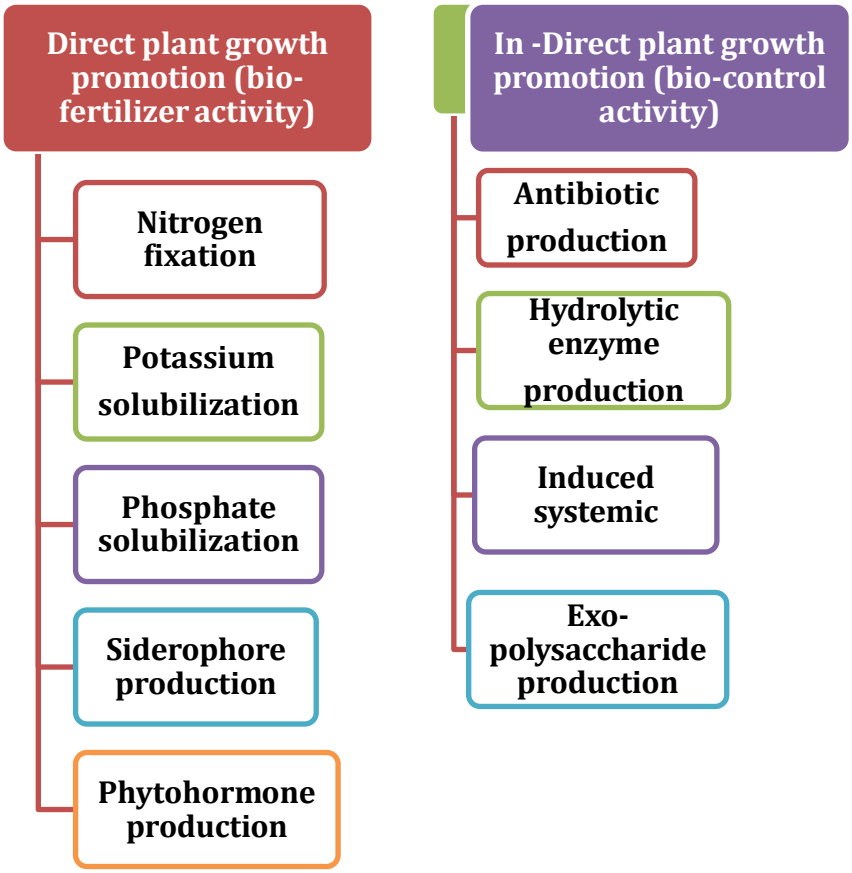
- Proficient to colonize the root surface
- Survive, multiply and compete with other micro biota
- Promote plant growth.

Nowadays, agricultural production is dependent on the large-scale use of chemical fertilizers. Chemical fertilizers have become integral and necessary components of modern agriculture because they provide essential plant nutrients (macro- as well as micronutrients) such as nitrogen (N), phosphorus (P), and potassium (K). However, the excess use of chemical fertilizers for increased crop production leads to harmful environmental impacts (Adesemoye et al. 2009). The use of efficient PGPR inoculants is an important strategy to achieve maximum benefits in terms of fertilizer savings and better growth and for reducing environmental problems caused by the use of chemical fertilizers (Hungria *et al.*, 2013)

Role and importance of PGPRS in agriculture

Plant growth promoting rhizobacteria promote plant growth directly and indirectly.





Mechanism of action of PGPR

Direct mechanisms

Most of the agricultural soils suffer from the deficiency of one or more essential nutrients. Deficiency of essential nutrients in soil made the soil unsuitable for crop production as plant growth will be suboptimal. To get higher productivity and reduce this problem, farmers are

extensively using chemical fertilizers as the sources of macro- as well as micronutrients (especially nitrogen and phosphorous). Chemical fertilizers are expensive and their production depletes natural resources. Indiscriminate use of chemical fertilizers also poses human and environmental hazards. It would be economical and advantageous, if efficient biological means can be used to provide essential nutrients to plants. By this way, we can substitute for at least a portion of the chemical fertilizers that is currently used. These mechanisms positively influence the plant growth activity directly. So, the direct mechanism of PGPR is the major step involved to support growth and development of plants.

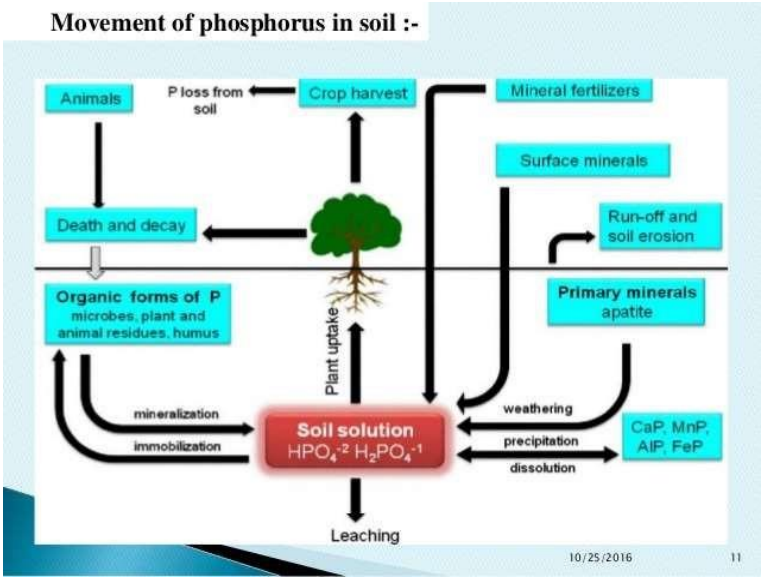
Nitrogen fixation

- PGPR fix atmospheric nitrogen and provide it to plants by two mechanisms.
- Symbiotic nitrogen fixation: mutual relationship between microbe and the plant.
- Non-symbiotic nitrogen fixation: is carried out by free living diazotrophs.
- Biological nitrogen fixing PGPR help in disease management growth promoting activity, maintain nitrogen level in agricultural soil.

Phosphate solubilization

Phosphorous is the most important key element in the nutrition of plants. PGPR convert insoluble phosphorus to an accessible form. Phosphate solubilization mechanisms employed by PGPR:

1) Release of complexing or mineral dissolving compounds
2) Liberation of extracellular enzymes
3) The release of phosphate during substrate degradation
4) PGPR act as inoculums to improve yield and Growth



Potassium solubilization

- Potassium is the third major essential macronutrient
- PGPR solubilize potassium rock through production and secretion of organic acids.

- Potassium solubilizing PGPR are *Acidothiobacillus ferrooxidans, Bacillus edaphicus* etc.

Siderophore production

- Siderophore is an iron-chelating compound help in assimilation of iron.
- Siderophore have been implicated for both direct and indirect enhancement of plant growth.

Phyto-hormone production

- Phyto-hormones such as auxins, cytokinins, gibberellins and ethylene affect cell proliferation.

Indole acetic acid production

- Produced by PGPR
- Help in plant cell division and differentiation
- Stimulate seed and tuber germination
- Biosynthesis of various metabolites, and resistance to stressful conditions.
- *Pseudomonas, Rhizobium, Bradyrhizobium, Agrobacterium, Enterobacter* and *Klebsiella* are IAA-producing PGPR.

Cytokinins and gibberelins

- PGPR *Azotobacter* sp., *Rhizobium* sp., *Bacillus subtilis* etc. can produce gibberellins both or can produce either cytokinin or gibberellins for plant growth promotion.
- It appears that PGPR produce lower cytokinin levels compared to phytopathogens.

Indirect mechanisms

The major indirect mechanism of plant growth promotion by probiotic rhizobacteria is through acting as biocontrol agents (Glick 2012). There are various modes of biocontrol activity in plant probiotics. It includes antibiotic production, competition for nutrients, niche exclusion, antifungal metabolite production, and induced systemic resistance. Many rhizobacteria with probiotic potential have been reported to produce antifungal metabolites like hydrogen cyanide, pyrrolnitrin, DAPG (2,4-diacetylphloroglucinol), pyoluteorin, and tensin (Bhattacharyya and Jha, 2012).

Interaction of some plant probiotics with the roots of plant can result in plant resistance against some pathogenic organisms which are harmful to plants. This phenomenon is called induced systemic resistance (Lugtenberg and Kamilova, 2009). Thus, plant growth-promoting rhizobacteria function as probiotics for plants as they improve the plant growth and productivity by combination of various direct and indirect mechanisms.

PGPR is a promising sustainable and environmentally friendly approach to obtain sustainable fertility of the soil and plant growth indirectly.

a) Antibiosis

- Antibiotics production is bio-control mechanisms of PGPR against phyto-pathogens.

- Increased use antibiotic PGPR as bio-control agent develop resistance against antibiotic.

b) Lytic enzymes

- PGPR strains produce enzymes such as chitinases, dehydogenase, β-glucanase, lipases etc. exhibit hyper parasitic activity.
- Protecting from biotic and abiotic stresses.

c) Induced and systemic resistance

It is a physiological state of enhanced defensive capacity elicited in response to specific environmental stimuli and consequently the plants innate defences are potentiated against subsequent biotic challenges.

d) Exo-polysachharide production

- Production of Exo-polysachharide is important in biofilm formation and root colonization.
- Effective colonization of plant roots by EPS-producing microbes helps to hold the free phosphorus and circulating essential nutrient to the plant.
- Functions performed by EPS producing microbes constitute shielding from desiccation, attachment to surfaces plant invasion, and plant defence responses in plant-microbe interactions.

Impact of PGPR on root system architecture and root structure

Most terrestrial plants develop their root system to explore soil and find nutrients to sustain growth. In fabaceae for example, the root tip help in initiating the rhizobial colonization process. In poaceae, rot hairs and lateral roots are colonized by PGPR. RSA intergrated root system topology, the spatial distribution of primary and lateral roots, and the number and the length of various types of roots.

PGPR as biofertilizers

- The search for PGPR and investigation of their modes of action increasing at rapid pace as efforts are made to exploit them commercially as biofertilizers.
- PGPR help in fixing N2, increasing the availability of nutrients, positively influencing root growth and morphology, and promoting other beneficial plant-microbe symbioses.
- The combination of these modes actions in PGPR is also addressed and widespread utilization of PGPR as biofertilizers.

Bio-control properties of PGPRS

Bio-control is a process through which a living organism limits the growth. The mechanisms of bio-control by rhizobia include competition for nutrients, production of antibiotics, and production of enzymes. Rhizobial strains compete for nutrients by displacing the pathogens. Rhizobia starve the pathogens of available iron by producing high affinity siderophore.

Influence of PGPR on nutrient uptake

- The combined application of *Azospirillum, phosphobacteria* and VAM with 75% of recommended NPK recorded higher yield of potato (14.96t/ha).

- The inoculation of *Rhizobium, P. striata* significantly increased nitrogen and phosphorus uptake by chickpea over control.
- The highest N and P uptake was recorded in Rhizobium +20 kg N and 60kg P per ha.
- The promotion mechanism by rhizobial strains is production of IAA, resulted in the increase of the plant growth.
- An increase in absorption of water and nutrients from soil has been observed in tomato when PGPR has been inoculated.

Impact of PGPR on soil health

To meet the food demands of increasing human population, the crop productivity needs to be increased. But due to decrease in the cultivable land due to rapid urbanization, farmers are dependent on fertilizers to get higher productivity. For this, they use chemical fertilizers which degrade soil quality. The inoculation of beneficial rhizobacteria not only improves plant growth and yield attributes but it also improves the soil health. The beneficial rhizobacteria on inoculation improve the nutrient status of soil by providing essential nutrients as they are involved in transformation of nutrients. Plant growth-promoting rhizobacteria fix atmospheric nitrogen, solubilize organic phosphorous, and detoxify heavy metals. These traits of PGPR help them to improve soil health.

Effect of inoculation of probiotic PGPR on soil biological activities

The plant growth-promoting rhizobacteria act as probiotic both for plant and soil because in addition to improving plant health they also improve the fertility and biological activity of soil. The effect of inoculation of PGPR as probiotics along with the differential doses of inorganic fertilizers and organic manures was studied on soil biological activities in case of potato crop. It was observed that the application of various organic sources and beneficial rhizobacteria significantly improves microbial population, soil enzyme activity, and physicochemical properties of soil, which in turn improved the nutrient uptake, yield attributes, and yield of potato crop

Future research and development strategies for sustainable technology

- The need of today's world is high output yield and enhanced production of the crop as well as fertility of soil to get in an eco-friendly manner.
- Future research in rhizosphere biology will rely on the development of molecular and biotechnological approaches to increase our knowledge of rhizosphere biology.
- Fresh alternatives should be explored for the use of bio-inoculants.
- The application of multi strain bacterial consortium over single inoculation could be ineffective approach for reducing the harmful impact of stress on plant growth.
- Research on nitrogen fixation and phosphate solubilization by PGPR is progress on but research done on potassium solubilization is little, will not only increase the field of the inoculants but also create confidence among the farmers for their use.
- Higher yield and cost effective PGPR products use by agricultural farmer.

Conclusion:

PGPR are economically and environmentally beneficial for plant growth promotion. PGPR may have a direct or an indirect mode of action. PGPR may function as bio fertilizer, bio-inoculant, and other growth promoting activity. New concept need to be constantly developed.

References:

1. Adesemoye AO, Torbert HA, Kloepper JW (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microb Ecol 58:921–929
2. Ahemad M, Kibret M (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ Sci
3. Bhattacharyya PN, Jha DK (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350
4. Dakora FD, Phillips DA (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245:35–47
5. Glick B (2012). Plant growth-promoting bacteria: mechanisms and applications. Scientifica 2012:1–15
6. Hiltner L (1904). Over recent experiences and problems in the field of soil bacteriology and special those into account the Grundungung and fallow. Arb Deutsche Agricultural Enges 98:59–78
7. Hungria M, Nogueira MA, Araujo RS (2013). Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. Biol Fertil Soils 49:791–801
8. Kang BG, Kim WT, Yun HS, Chang SC (2010). Use of plant growth-promoting rhizobacteria to control stress responses of plant roots. Plant Biotechnol Rep 4:179–183
9. Kloepper JW, Leong J, Teintze M, Schroth MN (1980). Enhancing plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885–886
10. Lugtenberg B, Kamilova F (2009). Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556

UNDERSTANDING DIFFERENT TYPES OF VACCINE

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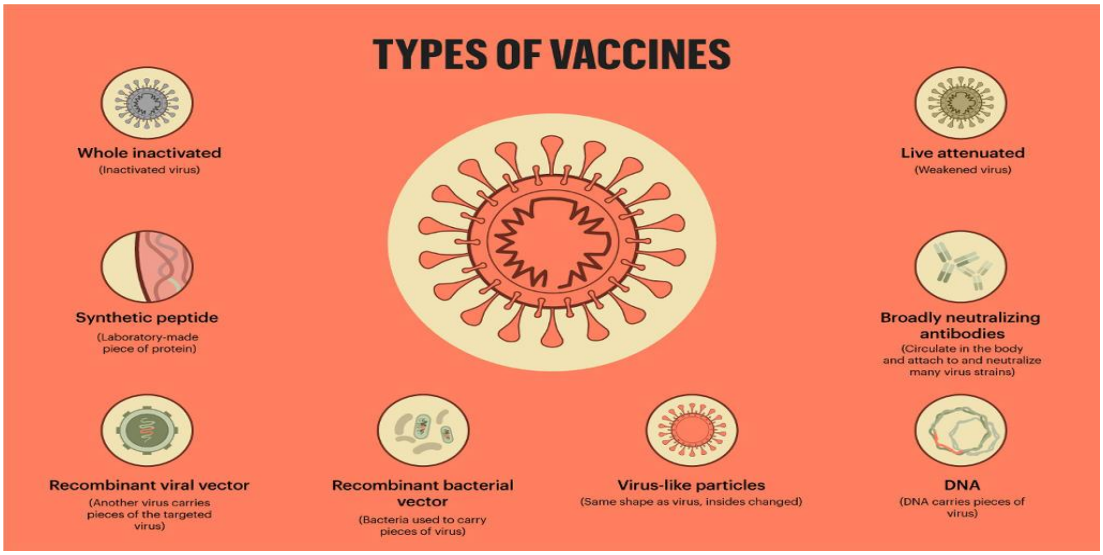
A vaccine is a biological preparation that provides active acquired immunity to a particular infectious. A vaccine typically contains an agent that resembles a disease-causing microorganism and is often made from weakened or killed forms of the microbe, its toxins, or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as a threat, destroy it, and recognize further and destroy any of the microorganisms associated with that agent that it may encounter in the future.

The administration of vaccines is called vaccination. Vaccination is the most effective method of preventing infectious diseases.

Types of vaccines:

Vaccines typically contain attenuated, inactivated or dead organisms or purified products derived from them. The types of vaccine are as follows:



Types of Vaccines

A. Whole organism vaccines:

Whole organism vaccine consists of two types of vaccines: Live attenuated and Heat killed or Inactivated vaccines.

Live attenuated: Some vaccines contain live, attenuated microorganisms. Many of these are active viruses that have been cultivated under conditions that disable their virulent properties, or that use closely related but less dangerous organisms to produce a broad immune response. Although most attenuated vaccines are viral, some are bacterial in nature.

Examples:

- Measles, mumps, rubella (MMR combined vaccine)

- Rotavirus
- Smallpox
- Chickenpox
- Yellow fever
- Polio (Sabin vaccine)
- Varicella
- BCG (*Mycobacterium tuberculosis* vaccine)

Advantages: Strong immune response; often lifelong immunity with few doses. Attenuated, or live, weakened, vaccines typically provoke more durable immunological responses.

Disadvantages: On rare occasions mutate to a virulent form and cause disease. Requires refrigerated storage.

Inactivated or killed vaccines: Another common means to achieve attenuation of a vaccine is inactivation of the pathogen by heat or chemical means so that the pathogen raises an immune response but is not capable of replication in the host.

Examples:

- Cholera
- Plague
- Polio (Salk vaccine)
- Hepatitis A vaccine
- Rabies vaccine
- Influenza vaccines.

Advantages: Stable; safer than live vaccine. Refrigerated storage not required.

Disadvantages: Weaker immune response than live vaccines. Booster shots usually required.

B. Purified macromolecules: Which do not include whole organism but only the major antigenic part of the pathogen.

Toxoid (Inactivated exotoxin): Toxoid vaccines are made from inactivated toxic compounds that cause illness rather than the micro-organism.

Examples:

- Tetanus
- Diphtheria
- Pertussis (whooping cough) vaccine

Advantage: Immune system become primed to recognize bacterial toxins.

Subunit vaccines: Many of the risks associated with attenuated or killed whole organism vaccines can be avoided with vaccines that consist of specific, purified macromolecules derived from pathogens like inactivated exotoxins, capsular polysaccharides, and recombinant protein antigens.

Examples: Hepatitis B, which is composed of only the surface proteins of the virus. *Streptococcal pneumoniae*, composed of capsular polysaccharides.

Advantages: Specific antigens lower the chance of adverse reactions.

Disadvantage: Difficult to develop.

Conjugate vaccines: Conjugate meaning to connect. With some bacteria, to get protection from a vaccine you need to train the immune system to respond to polysaccharides rather than proteins. But in the early days of polysaccharide vaccines, it was found that they did not work well in babies and young children. Polysaccharide was attached (conjugated) to something else that creates a strong immune response. In most conjugate vaccines, the polysaccharide is attached to diphtheria or tetanus toxoid protein. The immune system recognises these proteins very easily and this helps to generate a stronger immune response to the polysaccharide.

Examples:

Haemophilus influenzae type B

Streptococcal pneumonia

Advantages: Primes infant immune systems to recognize certain bacteria.

C. DNA vaccines: A vaccination strategy under investigation for a number of diseases utilizes plasmid encoding antigenic proteins, which is injected directly into the muscle of the recipient. Muscle cell take up the DNA and the encoded protein antigen is expressed, leading to both humoral and cell mediated responses. DNA is more stable than mRNA so doesn't require the same initial protection. DNA vaccines are typically administered along with a technique called electroporation. This uses low level electronic waves to allow the bodies' cells to take up the DNA vaccine. DNA must be translated to mRNA within the cell nucleus before it can subsequently be translated to protein antigens which stimulate an immune response. **Advantages**: Strong humoral and cellular response. Relatively inexpensive to manufacture

D. Recombinant vector vaccines: Genes that encode major antigens of virulent pathogens can be introduced into attenuated viruses or bacteria. The attenuated organism serves as a vector, replicating within the host and expressing the gene product of the pathogen. A number of organisms have been used for vector vaccines, including vaccinia virus, adenoviruses, attenuated polio viruses, attenuated strains of *Salmonella*, the BCG strain of *Mycobacterium bovis*.

Advantages: Mimics natural infection, resulting in strong immune response.

References:

1. https://en.wikipedia.org/wiki/Vaccine
2. https://www.hhs.gov/immunization/basics/types/index.html
2. https://www.niaid.nih.gov/research/vaccine-types
3. https://vaccineknowledge.ox.ac.uk/types-of-vaccine
4. https://vaccineknowledge.ox.ac.uk/covid-19-oxford-vaccine-trial-infographic
5. Reference book: Immunology - Kuby 6th edition

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